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Clinical application of liquid biopsy to identify predictive and resistance biomarkers in stage IV breast cancer patients treated with CDK4/6i

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CLINICAL APPLICATION OF LIQUID BIOPSY TO IDENTIFY PREDICTIVE AND RESISTANCE BIOMARKERS IN STAGE IV BREAST CANCER PATIENTS TREATED WITH CDK4/6 INHIBITORS

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Seize the day!

Don’t stop believing

A ***Celso,***

Sobran las palabras,
esta tesis es tan tuya como mía.

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ABBREVIATIONS

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AUC	Area under curve
BC	Breast cancer
BCME	Breast cancer microenvironment
CDK4/6i	Cyclin-dependent kinase 4/6 inhibitor
cDNA	complementary DNA
cfDNA	Cell-free DNA
CDR3M	Cell-free DNA ratio at 3 months
CDR6M	Cell-free DNA ratio at 6 months
CTCs	Circulating tumor cells
ctDNA	Circulating tumor DNA
DC	Dendritic cells
ddPCR	Digital-droplet PCR
DGE	Differential Gene Expression
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EMA	European Medicine Agency
EMT	Epithelial mesenchymal transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
ET	Endocrine therapy
EV	Extracellular vesicles
FBS	Fetal Bovine serum
FDA	Food and Drug Administration
GO	Gene Ontology
HER2	Human Epidermal growth factor Receptor 2
HR	Hormonal Receptor
MCF7p-R	MCF7 palbociclib resistant
MHC	Major histocompatibility complex
miRNA	micro RNA
MTT	Thiazolyn Blue Tetrazolium Bromide
NGS	Next Generation Sequencing
NK	Natural killers
OS	Overall survival
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphatase Bovine Serum
PDX	Patients Derived Xenografts
PFS	Progression free survival
PR	Progesterone Receptor
ROC	Receiver operating characteristic curve
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse Transcription Quantitative PCR
T47Dr-R	T47D ribociclib resistant
TAMS	Tumor-associated macrophages
TILS	Tumor infiltrating lymphocytes
TN	Triple negative
TNM	Tumor Node Metastasis Staging system

RESUMO

RESUMO

O cancro de mama (CM) é o máis común entre as mulleres, sendo a enfermidade avanzada a principal causa de morte. A incidencia e mortalidade deste carcinoma aumentou nos últimos anos por diferentes cambios biolóxicos e socio-culturais. Describese como unha enfermidade heteroxénea cuxo prognóstico depende das características anatómicas, da cualificación TNM e do subtipo de cancro. Ata o de agora, o cancro de mama clasifícase en tres subtipos segundo a expresión do receptor 2 do factor de crecemento epidérmico humano (HER2) e a expresión de dous receptores hormonais (HR): receptor de estrógeno (ER) e receptor de proxesterona (PR). O subtipo HR ou luminal caracterízase por expresar receptores hormonais. É o subtipo máis común e menos agresivo, dividíndose en luminal A ou B segundo o cociente de proliferación e a expresión de HER2. O subtipo HER2 puro caracterízase pola sobreexpresión de HER2. Por último, o subtipo triplo negativo (TN) non expresa os marcadores anteriores. É o subtipo máis agresivo con peor prognóstico. O réxime de terapia para o subtipo HR+ é a terapia endocrina (ET), para o subtipo HER2 son terapias dirixidas a HER2 mentres que para o subtipo TN a quimioterapia ou inmunoterapia.

Sobre o 70 % dos doentes con cancro de mama son diagnosticadas co subtipo HR+/HER2-. Independentemente do estadio (temperán ou avanzado), este subtipo tratouse con terapia endocrina, pero os estadios avanzados normalmente adquiren resistencia á terapia endocrina. Por iso, a FDA aprobou a combinación dos inhibidores de quinasas 4/6 dependentes de ciclinas (CDK4/6i) xunto con ET como primeira liña metastática para tratar os doentes con cancro de mama avanzado subtipo HR+/HER2-. Os inhibidores de CDK4/6 dispoñibles comercialmente son Palbociclib, Ribociclib e Abemaciclib. Este enfoque terapéutico mellora a supervivencia en máis de dous anos con escasos efectos secundarios, como foi demostrado nos ensaios clínicos PALOMA, MONALEESA e MONARCH. Con todo, un 20 % das doentes non responden a terapia combinada debido a resistencia intrínseca. Ademais, aqueles doentes que inicialmente responden terminan desenvolvendo resistencia adquirida. Como a biopsia de tumor realizouse anos antes de que a enfermidade progresase, non reflicte as características tumorais actuais. Ademais, a biopsia das metástases non sempre é posible. Por tanto, para determinar que doentes se beneficiarán da combinación de CDK4/6i e ET, as características tumorais actuais deberían estudarse, sendo a biopsia líquida unha boa estratexia.

A biopsia liquida defínese como a mostraxe e análise do material derivado do tumor en diferentes fluídos biolóxicos como o sangue, saliva ou ouriños. Esta aproximación supera as desvantaxes da biopsia de tecido xa que permite estudar o tumor de maneira repetitiva, a tempo real e de forma non invasiva. A combinación da información obtida de ambas biopsias, podería

mellorar o manexo dos doentes en termino de diagnóstico temperán, predición de resposta á terapia, detección de resistencia, identificación de dianas terapéuticas etc. O material derivado do tumor máis estudado mediante a biopsia líquida é o DNA tumoral circulante (ctDNA), pero recentemente estase ampliando ás células tumorais circulantes (CTCs), microRNAs (miRNAs), RNA tumoral circulantes e vesículas extracelulares (EVs).

O DNA celular libre (cfDNA) libérase á circulación polos tecidos apoptóticos e as células hematolóxicas en condicións fisiolóxicas. A súa vida media varia entre 16 minutos e 2.5 horas. O ctDNA é unha pequena fracción do cfDNA (< 1%) liberado do tumor primario, metástase ou CTCs. A cantidade de ctDNA detectada na circulación depende do número de células mortas, metabolismo tumoral, necrosis, apoptosis etc. Úsase na clínica para rastrexar o perfil mutacional ou detectar enfermidade residual. Con todo, as CTCs están a gañar importancia como material derivado do tumor no campo oncolóxico. Estas células son células tumorais intactas que reflicten as características moleculares tumorais actuais. As CTCs libéranse ao torrente sanguíneo desde o tumor primario ou metástase activamente ou pasivamente, sendo capaces de formar outras metástases. As CTCs son extremadamente raras en circulación, 1-10 células únicas ou en agrupacións por 10 mL de sangue, sendo a súa vida media entre 1 e 2.4 horas. A súa curta vida media xunto coa súa escaseza complica o seu illamento e caracterización. O principal desafío en oncoloxía é determinar que subpoboación de CTCs inicia a lesión metastática. Para iso, os métodos de illamento e detección deberían evitar a limitación introducida cando se basean na expresión de marcadores ou características físicas. Actualmente, está aprobado a contaxe de CTCs polo sistema CellSearch® como informativo do prognóstico do doente, pero este enfoque non informa sobre as características tumorais, necesarias para a toma de decisións. Como consecuencia, é importante estudar o transcriptoma, proteoma e epixenomica das CTCs para comprender a paisaxe xenética do tumor. Ademais, está a gañar importancia a xeración de modelos de CTCs para estudar os procesos relacionados co desenvolvemento do cancro, formación da metástase ou comprobar a eficacia e seguridade de fármacos, para así realizar unha medicina de precisión.

En canto á adquisición de resistencia nas doentes con cancro avanzado subtipo HR+/HER2-, varios mecanismos de resistencia a CDK4/6i foron propostos, estudándose principalmente no ctDNA. Como CDK4/6i e a ET actúan no mesmo eixo, certos mecanismos de resistencia son comúns, o que complica o estudo da resistencia á combinación de ambos fármacos. Os mecanismos de resistencia propostos agrúpanse como específicos de ciclo celular, por exemplo, perdida de *RB1*, alteración dos inhibidores intrínsecos de *CDK*, amplificación en *CCND1* ou *CDK4/6* etc., así como non específicos de ciclo celular como a activación de eixos de sinalización (ER1 ou PI3K/AKT/mTOR), aberracións en *FGFR1*, transición epitelio-mesénquimal etc. Con todo, ningún mecanismo de resistencia foi validado. En conxunto, o desafío clínico-oncolóxico actual é manexar as doentes con cancro avanzado e subtipo HR+/HER- resistentes á terapia combinada. Neste contexto, o obxectivo desta tese é identificar marcadores relacionados con resposta e resistencia a CDK4/6i con ET caracterizando o material circulante dos doentes. Para iso, recolleuse sangue de doentes con cancro de mama avanzado subtipo HR+/HER2- en diferentes momentos: cando se diagnosticou a enfermidade metastática

(visita 1), despois dun ciclo de terapia (visita 2), no momento da progresión da enfermidade (visita 3) e cada 3 meses dende a visita 1 ata os 24 meses. Considerando o momento no que a enfermidade progresou, as doentes clasificáronse como non respondedoras ou con resistencia intrínseca se a progresión da enfermidade produciuse nos 6 meses seguintes á visita 1; e como respondedoras ou con resistencia adquirida se a progresión da enfermidade produciuse despois de 6 meses desde o inicio da terapia.

A partir da mostra de sangue periférico, illáronse as CTC cun método de enriquecemento negativo, o RosseteSep. A continuación, estas células caracterizáronse a nivel transcriptómico utilizando o panel PanCancer Pathway (tecnoloxía nCounter) para descubrir os xenes expresados diferencialmente entre doentes con diferente resposta terapéutica ou adquisición de resistencia. Ademais, desenvolveuse un modelo *in vitro* resistente a CDK4/6i para estudar a expresión de xenes relacionados coa resistencia, previamente identificados na nosa cohorte de doentes. Tamén se analizou o perfil mutacional das CTCs utilizando o panel Human Comprehensive e, a continuación, validáronse as mutacións en mostras de ctDNA mediante ddPCR. Ademais, mediuse de xeito lonxitudinal a concentración do cfDNA para monitorizar tanto a resposta ao tratamento como a progresión da enfermidade. Por último, comprobouse se os PBMCs poderían ser un biomarcador prognóstico do risco de cancro de mama e da resposta ao tratamento. Para iso, recolléronse mostras de sangue periférico de mulleres libres de cancro e de doentes con enfermidade temperá ou avanzada antes do inicio da terapia (visita 1). A continuación, illáronse PBMC usando Lymphoprep e analizouse a expresión xénica dun conxunto seleccionado de xenes en mulleres libres de cancro, con estadio temperán e avanzado de CM. Neste último grupo, tamén se estudou a expresión xénica entre doentes con diferente resposta terapéutica. Así mesmo, analizáronse xenes informativos sobre o risco de CM en doentes con CM avanzado TN. Ademais, realizouse un co-cultivo paracrino con PBMCs naïve ou de doentes HR+ cunha liña celular de cancro HR+ para estudar mediante RT-qPCR como a interacción entre ambos tipos celulares cambia a súa expresión xénica.

Incluíronse vinte e cinco doentes con CM en estadio avanzado co subtipo HR+/HER2- no estudo para identificar biomarcadores nas CTCs. A idade media no momento do diagnóstico da enfermidade avanzada foi de 60 anos. Todas as doentes recibiran CDK4/6i máis ET como terapia metastática de primeira liña. Existe un desequilibrio cara á administración de Palbociclib (68 % das doentes) na nosa cohorte. As doentes non respondedoras (n=5) progresaron nun tempo medio de 105 días, mentres que as doentes respondedoras (n=20) progresaron nun tempo medio de 601 días. Un total de 18 doentes sufriron progresión da enfermidade, mentres que 10 faleceron durante o seguimento. O illamento das CTCs con RosetteSep evitou a limitación introducida polos métodos baseados nas propiedades moleculares. Con este método enriquecéronse todas as poboacións de CTCs e, en concordancia, observouse un fenotipo epitelial-mesenquimal con expresión de marcadores de células nai nas visitas 1, 2 e 3. Ademais, a enumeración das CTCs mediante o sistema CellSearch® realizouse en 12 doentes na visita 1 e 2 mentres que en 7 doentes na visita 3, mostrando que as doentes con ≥ 1 CTC na visita 2 tiñan unha supervivencia máis curta.

Ademais, ter ≥ 1 CTC asocia cunha baixa expresión de *ALDH1A1* na visita 3, momento no que este xene tamén asocia co peor prognostico. En canto á expresión xénica das CTCs, na visita 1 as doentes non respondedoras tiñan unha maior expresión dos xenes *STAT3*, *PRKCB* e *MAPK3*, mentres que as doentes respondedoras tiñan unha maior expresión dos xenes implicados na vía CCND1-CDK4/6. Así mesmo, a expresión elevada de *CCND1*, *DUSP5* e *GZMB* identifica que doentes terán unha resposta máis prolongada á terapia combinada (>2 anos). Ademais, a expresión de *DUSP5*, *CDK4* e *PALB2* asocia a un mellor prognóstico das doentes. Na visita 2, as doentes que non responderon tiñan unha maior expresión dos xenes *CDH1*, *CDKN1C* e *CUL1*, sendo *CDKN1C* tamén indicativo dun peor prognóstico. Na visita 3, *BAX*, *EZH2*, *PLAU*, *RELA*, *NFKBIα* e *SNAIL1* son informativos da resistencia intrínseca e adquirida a CDK4/6i máis ET. Curiosamente, a expresión de *HDAC6* e *CDC7* foi especificamente informativa de resistencia adquirida, mentres que os niveis elevados de *CASP8* atopáronse en doentes con resistencia intrínseca. Ademais, *ALDH1A1* e *STAT3* asociáronse coa peor supervivencia dos doentes. Non se atopou ningunha asociación entre a expresión xénica de CTC e as características clínicas das doentes (terapia recibida, localizacións metastásicas etc.).

Na visita 1, descubriuse que a expresión combinada de $STAT3^{alto}$-$PRCKB^{alto}$-$CDK6^{baixo}$ determina que doentes responderán á terapia combinada. Esta firma foi validada nunha cohorte interna (n=12), mostrando a mesma precisión na clasificación dase doentes. Ademais, a firma tamén foi validada nunha cohorte externa (n=16) na que as CTCs foron illadas con Adnatest, que se basea na expresión de marcadores de superficie (EpCAM, HER2 e EGFR), o que demostra que esta firma clasifica ás doentes independentemente do método de illamento ou procedemento das mostras.

Ademais, tamén se atopou unha asociación entre a expresión xénica das CTCs. Así, na visita 1 identificouse unha asociación entre os marcadores mesenquimales *SNAIL1* e *VIM* así como entre os marcadores de células nai *ALDH1A1* e *PLS3*. Tamén se atopou que a expresión de *PI3KCG* asociaba positivamente con *JAK2* e *CUL1* mentres que *STAT3* con *PRKCB*. Así mesmo, atopouse unha asociación positiva entre *CDK4* e *6*. Na visita 2, observouse unha asociación negativa entre *CDK4* e *CDKN1C*, mentres que unha asociación positiva entre *CCND1* con *CDKN2C* e *STAT3* con *MAPK3*. Por último, na visita 3 identificouse unha asociación entre a expresión de *STAT3* con *PRKCB*, *JAK2* e *ALDH1A1* e dous xenes apoptóticos, *BAX* e *CASP8*.

Xerouse un modelo de liña celular resistente utilizando as liñas celulares hormonais MFC7 e T47D para estudar a expresión de xenes relacionados coa resistencia a CDK4/6i na nosa cohorte en estadio avanzado. A liña celular MCF7 fíxose resistente a Palbociclib (MCF7p-R) mentres que a T47D a Ribociclib (T47Dr-R). A adquisición de resistencia confirmouse mediante citometría de fluxo, ensaios de proliferación e formación de colonias. O modelo MCF7p-R foi o mellor para estudar os xenes relacionados coa resistencia, xa que reflecte con exactitude os patróns de expresión observados nas doentes con CM en estadio avanzado.

Realizouse unha análise exhaustiva do ADN das CTCs para identificar alteracións relacionadas coa resposta ao tratamento. Todos os doentes compartían unha mutación patoxénica en *KMT2C* e *C/EBPα* na primeira visita. Curiosamente, todas as doentes non respondedoras incluídas presentaban mutacións de perda de función en ARID1A, que promove a progresión do ciclo celular xunto con mutacións no xene *C/EBPα*. Así mesmo, as doentes respondedoras tiñan unha depleción en *ARID1B*, que evita a progresión do ciclo celular. Tamén se atopou unha deleción no xene *HSP900A1* no ADN das CTC das doentes non respondedoras. Dado que tamén se atopou no ctDNA de todas as doentes HR+/HER2- en estadio avanzado, descartouse como biomarcador de resposta. Realizouse un estudo máis profundo analizando o estado mutacional de *PI3K* no ctDNA de doentes que progresaron a CDK4/6i máis ET, pero non se atopou ningunha asociación entre a presenza de mutacións en *PI3K* e a resposta á terapia combinada ou supervivencia da doente. Con todo, atopouse unha asociación entre ter *PI3K* alterada e alta expresión de *HDAC6*, así como *PI3K* wild-type e alta expresión de *CUL1*, *RAC2* e xenes apoptóticos (*BAX* e *CASP8*). Estes resultados sinalaron que a vía PI3K está alterada en doentes con CM en estadio avanzado, que afecta a outras vías tumorixénicas. Ademais, o estudo lonxitudinal do cfDNA permitiu monitorizar a evolución da enfermidade. Así mesmo, o cociente de cfDNA mostrou que as doentes respondedoras tiñan niveis de cfDNA máis baixos que os non respondedoras, o que tamén se asociaba coa supervivencia das doentes.

Incluíuse a un total de noventa e oito doentes para estudar o papel dos PBMC no CM. Incluíronse 20 mulleres libres de cancro, 21 HR+ e 13 TNBC en estadio temperán, así como 26 HR+/HER2- e 18 TNBC en avanzado. Estudando ambos subtipos, a idade media no momento do diagnóstico do tumor primario (estadio temperán) foi de 59 anos. Todas as doentes foron tratadas con quimioterapia. A idade media ao diagnóstico da enfermidade metastática foi de 61 anos. O subtipo HR+ foi tratado con CDK4/6i máis ET e desenvolveron metástase principalmente en óso e vísceras. O subtipo TNBC tratouse con quimioterapia e desenvolveu metástase principalmente nas vísceras. Os PBMC de doentes con CM en estadio avanzado illáronse con Lymphoprep antes do inicio da terapia. Observouse unha maior expresión de *GZMB*, *KLF4* e *MYCL* nas doentes respondedoras. A continuación, analizáronse os xenes expresados diferencialmente en PBMC de mulleres libres de cancro e en estadios iniciais de CM. *KITLG*, *KMT2D*, *KFL4* e *MYCL* expresáronse en maior medida nas mulleres sen cancro que nas doentes con CM. Curiosamente, a expresión de *KITLG* diminúe progresivamente durante a enfermidade, probablemente debido á transformación das células malignas e á alteración do microambiente mamario tumoral. Ademais, observouse a expresión de *GZMB*, unha proteína liberada polas células inmunitarias efectoras, tanto na fracción enriquecida de CTC como nos PBMCs. Estes resultados tamén se estudaron no subtipo TN, no que tamén se atopou unha maior expresión de *KITLG*, *KLF4* e *MYCL* nas mulleres libres de cancro.

Tras un co-cultivo paracrino, estudouse a expresión dos xenes mencionados, comprobándose que a expresión de *KITLG* e *KLF4* diminúe significativamente nos PBMCs naïve, pero non nos expostas previamente ao microambiente tumoral. Tamén se observou que os niveis de leucocitos diminuían tras un ciclo de terapia, pero non todos os tipos de leucocitos diminuían por igual en todos os grupos de doentes. Calculouse o cociente NLR como marcador

da resposta inmunitaria. No subtipo HR+, o NLR mostrou uns niveis de estrés fisiolóxico normais tras un ciclo de terapia, pero no TNBC segue habendo doentes con peores niveis de estrés fisiolóxico tras un ciclo de quimioterapia.

Considerando todos os resultados, pódese dicir que os nosos datos reforzan a utilidade clínica de utilizar as CTCs como marcadores prognósticos e preditivos para o manexo de doentes con CM estadio avanzado e subtipo HR+/HER2-. Demostrouse que a metodoloxía de enriquecemento negativo permite o illamento de diferentes subpoblacions de CTCs, xa que se detectaron CTCs con fenotipo epitelial e mesenquimal en diferentes puntos temporais. Con todo, debe terse en conta a contaminación con linfocitos non eliminados na análise de CTCs. No noso caso, a expresión de *GZMB* se correlaciona coa resposta terapéutica cando se analizan as CTC, pero trátase dun xene liberado por efectores celulares inmunitarios.

Tras a análise das CTC, confírmase que o número de CTCs en sangue periférico permite determinar o prognóstico das doentes. Ademais, varios biomarcadores identificados nas CTCs poderían guiar a selección da terapia nas visitas 1 e 3. Antes de iniciar a terapia, describiuse un aumento da expresión de *STAT3*, *PRKCB* e *MAPK3* que promove a resistencia a CDK4/6i máis ET na enfermidade avanzada. De feito, xa se desenvolveron inhibidores de *STAT3* e *MAPK3*, cuxa eficacia se está probando en combinación con CDK4/6i. *DUSP5* está altamente expresado en doentes respondedoras, inhibindo a activación de *MAPK3*. Así pois, a expresión conxunta de ambos os xenes debe terse en conta antes de iniciar a terapia. Así mesmo, tamén se observou que a resposta máis prolongada á terapia combinada prodúcese en doentes con alta expresión de xenes implicados no eixo *CCND1-CDK4/6*. É importante destacar que a firma desenvolvida considerando a expresión de $STAT3^{alto}$-$PRKCB^{alto}$- $CDK6^{baixo}$ determina con precisión que doente con cancro de mama estadio avanzado subtipo HR+/HER2 beneficiarase da terapia combinada, independentemente do método de illamento de CTC utilizado. Na progresión da enfermidade, observouse que varios mecanismos epixenéticos estaban alterados, como *EZH2* e *HDAC6*, por iso é polo que deba estudarse o perfil epixenético das CTC. Están a probarse inhibidores contra *EZH2* e *HDAC6* en varios cancros que teñan *ARID1A* mutado, unha alteración identificada nas doentes non respondedoras. De feito, estudouse a combinación dun inhibidor de *HDAC6* con inhibidores de PI3K ou CDK4/6 como terapia combinada para doentes con CM estadio avanzado subtipo HR+/HER2-. Ademais, a elevada expresión de *CDC7* demostrou que a progresión do ciclo celular non depende do eixo CCND1-CDK4/6 na adquisición de resistencia. Así mesmo, é importante o papel dual de *CDK6*, xa que na progresión da enfermidade promove o crecemento tumoral a través da interacción cos factores *NF-kB*. Evitando a súa interacción, restablécese a sensibilidade CDK4/6i. Algúns dos xenes antes mencionados relacionados coa resistencia foron validados só na liña celular MCF7p-R, o que pon de manifesto a importancia de considerar as propiedades xenéticas e físicas dos modelos seleccionados. Unha das terapias alternativas tras a resistencia a CDK4/6i son os inhibidores de PI3K. Observouse que as mutacións de *PI3K* no ctDNA da nosa cohorte eran frecuentes nas doentes respondedoras e correlacionou con xenes cuxa expresión estaba alterada nos CTCs, como *CUL1*, que interacciona con *EZH2*, e *HDAC6*. Ademais, a alteración dos xenes relacionados con apoptosis (*BAX* e *CASP8*) atopouse na visita 3 e tamén correlacionou coa

mutación de *PI3K*. Ademais do estudo do ctDNA, os niveis de cfDNA permitiron clasificar ás doentes en función da resposta terapéutica aos 3 e 6 meses. Por último, o estudo do transcriptoma de PBMCs pode utilizarse para coñecer a gravidade da enfermidade. Por exemplo, prodúcese unha perda progresiva de *KITLG* a través da transformación maligna e a expresión de *KLF4* pérdese na enfermidade avanzada. Do mesmo xeito, estas células tamén informan sobre a resposta á terapia, xa que se atopou unha baixa expresión de *KLF4*, *MYCL* e *GZMB* nas doentes non respondedoras. De feito, a expresión de *GZMB* utilizouse para predicir a resposta ao tratamento en varios tipos de cancro. Con todo, é necesario seguir caracterizando a expresión de cada tipo de leucocito e estudar como cambian os seus niveis en función do réxime terapéutico. Ademais, confirmouse que a interacción entre PBMC e as células cancerosas afecta á expresión das células inmunitarias e, por tanto, á resposta inmunitaria.

Por tanto, tendo en conta todos os resultados, demóstrase a necesidade de combinar a información transcripcional, mutacional e epixenética das CTCs, así como os datos doutro material derivado do tumor para comprender a bioloxía tumoral e guiar a terapia dos doentes. Neste sentido, os nosos resultados mostraron que o estudo de CTCs, cfDNA e PBMCs proporciona información clinicamente relevante para o manexo das doentes con CM estadio avanzado e subtipo HR+/HER2- antes do inicio da terapia ou na progresión da enfermidade. Por tanto, requírese unha maior caracterización molecular deste material circulante nunha cohorte máis grande e en ensaios clínicos para complementar a información clínica para conseguir unha medicina de precisión.

RESUMEN

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El cáncer de mama (CM) es el más común entre las mujeres, siendo la enfermedad avanzada la principal causa de muerte. La incidencia y mortalidad de este carcinoma aumentó en los últimos años por diferentes cambios biológicos y socio-culturales. Se define como una enfermedad heterogénea cuyo pronóstico depende de las características anatómicas, de la calificación TNM y del subtipo de cáncer. Hasta ahora, el cáncer de mama se clasifica en tres subtipos según la expresión del receptor 2 del factor de crecimiento epidérmico humano (HER2) y la expresión de dos receptores hormonales (HR): receptor de estrógeno (ER) y receptor de progesterona (PR). El subtipo HR o luminal se caracteriza por expresar receptores hormonales. Es el subtipo más común y menos agresivo, dividiéndose es luminal A o B según la ratio de proliferación y la expresión de HER2. El subtipo HER2 puro se caracteriza por la sobreexpresión de HER2. Por último, el subtipo triple negativo (TN) no expresa los marcadores anteriores. Es el subtipo más agresivo con peor pronóstico. El régimen de terapia para el subtipo HR+ es la terapia endocrina (ET), para el subtipo HER2 son terapias dirigidas a HER2 mientras que para el subtipo TN la quimioterapia o inmunoterapia.

Sobre el 70 % de los pacientes con cáncer de mama son diagnosticadas con el subtipo HR+/HER2-. Independientemente del estadio (temprano o avanzado), este subtipo se trata con terapia endocrina, pero los pacientes con estadios avanzados normalmente adquieren resistencia a la terapia endocrina. Por ello, la FDA aprobó la combinación de los inhibidores de quinasas 4/6 dependientes de ciclinas (CDK4/6i) junto con ET como primera línea metastásica para tratas a las pacientes con cáncer de mama avanzado subtipo HR+/HER2-. Los inhibidores de CDK4/6 disponibles comercialmente son Palbociclib, Ribociclib y Abemaciclib. Este enfoque terapéutico mejora la supervivencia en más de dos años con escasos efectos secundarios, como fue demostrado en los ensayos clínicos PALOMA, MONALEESA y MONARCH. Sin embargo, un 20 % de los pacientes no responden a la terapia combinada debido a la resistencia intrínseca. Además, aquellos pacientes que inicialmente responden terminan desarrollando resistencia adquirida. Como la biopsia de tumor puede realizarse años antes de que la enfermedad progrese, no refleja las características tumorales en tiempo real. Además, la biopsia de las metastásicas no siempre es posible. Por lo tanto, para determinar que pacientes se beneficiarán de la combinación de CDK4/6i y ET, las características tumorales actuales deberían estudiarse, siendo la biopsia líquida una buena estrategia.

La biopsia liquida se define como el muestreo y análisis del material derivado del tumor en diferentes fluidos biológicos como la sangre, saliva u orina. Esta aproximación supera las

desventajas de la biopsia de tumor ya que permite estudiar el tumor de manera repetitiva, a tiempo real y de forma no invasiva. La combinación de la información obtenida de ambas biopsias, mejorará el manejo de los pacientes para el diagnóstico temprano de la enfermedad, predicción de la respuesta a terapia, detección de resistencia, identificación de dianas terapéuticas etc. El material derivado del tumor más estudiado mediante la biopsia líquida es el DNA tumoral circulante (ctDNA), pero recientemente se está ampliando a las células tumorales circulantes (CTCs), microRNAs (miRNAs), RNA tumoral circulantes y vesículas extracelulares (EVs).

El DNA libre celular (cfDNA) se libera a la circulación por los tejidos apoptóticos y las células hematológicas en condiciones fisiológicas. Su vida media varía entre 16 minutos y 2.5 horas. El ctDNA es una pequeña fracción del cfDNA (< 1%) liberado del tumor primario, metástasis o CTCs. La cantidad de ctDNA detectada en la circulación depende del número de células muertas, metabolismo tumoral, necrosis, apoptosis, etc. Se usa en la clínica para rastrear el perfil mutacional o detectar enfermedad residual. Sin embargo, las CTCs están ganando importancia como material derivado del tumor en oncología. Estas células son células tumorales intactas que reflejan las actuales características moleculares tumorales. Las CTCs se liberan al torrente sanguíneo desde el tumor primario o metástasis activamente o pasivamente, siendo capaces de formar otras metástasis. Las CTCs son extremadamente raras en circulación, 1-10 células individuales o en agrupaciones por 10 mL de sangre, siendo su vida media entre 1 y 2.4 horas. Su corta vida media junto con su escasez complica su aislamiento y caracterización. El principal desafío en oncología es determinar que subpoblación de CTCs inicia la lesión metastásica. Para ello, los métodos de aislamiento y detección deberían evitar el sesgo introducido cuando se basan en la expresión de marcadores o características físicas. Actualmente, está aprobado el contaje de CTCs por el sistema CellSearch® como informativo del pronóstico del paciente, pero este enfoque no informa sobre las características tumorales, necesarias para la toma de decisiones. Como consecuencia, es importante estudiar el transcriptoma, proteoma y epigenómica de las CTCs para comprender el paisaje genético del tumor. Además, está ganando importancia la generación de modelos de CTCs para estudiar los procesos relacionados con el desarrollo del cáncer, formación de la metástasis o comprobar la eficacia y seguridad de fármacos, para así aplicar una medicina de precisión.

En cuanto a la adquisición de resistencia en las pacientes HR+/HER2- con cáncer avanzado, varios mecanismos de resistencia a CDK4/6i han sido propuestos, estudiándose principalmente en el ctDNA. Como CDK4/6i y la ET actúan en la misma ruta, ciertos mecanismos de resistencia son comunes, lo que complica el estudio de la resistencia a la combinación de ambos fármacos. Los mecanismos de resistencia propuestos se agrupan como específicos de ciclo celular, por ejemplo, pérdida de RB1, alteración de los inhibidores intrínsecos de CDK, amplificación en CCND1 o CDK4/6, etc., así como no específicos de ciclo celular como activación de rutas de señalización (ER1 o PI3K/AKT/mTOR), aberraciones en FGFR1, transición epitelio mesenquimal, etc. Sin embargo, ningún mecanismo de resistencia ha sido validado.

En conjunto, el desafío clínico-oncológico actual es manejar las pacientes con cáncer avanzado y subtipo HR+/HER- resistentes a la terapia combinada. En este contexto, el objetivo de esta tesis es identificar marcadores relacionados con respuesta y resistencia a CDK4/6i y ET caracterizando el material circulante de esos pacientes. Para ello, se recogió sangre de pacientes con cáncer de mama avanzado subtipo HR+/HER2- en diferentes momentos: cuando se diagnosticó la enfermedad metastásica (visita 1), después de un ciclo de terapia (visita 2), en el momento de la progresión de la enfermedad (visita 3) y cada 3 meses desde la visita 1 hasta los 24 meses. Considerando el momento en el que la enfermedad progresó, los pacientes se clasificaron como no respondedores o con resistencia intrínseca si la progresión de la enfermedad se produjo en los 6 meses siguientes a la visita 1; y como respondedores o con resistencia adquirida si la progresión de la enfermedad se produjo después de 6 meses desde el inicio de la terapia.

A partir de la muestra de sangre periférica, se aislaron las CTC con un método de enriquecimiento negativo, el RosseteSep. A continuación, estas células se caracterizaron a nivel transcriptómico utilizando el panel PanCancer Pathway (tecnología nCounter) para descubrir los genes expresados diferencialmente entre pacientes con diferente respuesta terapéutica o adquisición de resistencia. Además, se desarrolló un modelo *in vitro* resistente a CDK4/6i para estudiar la expresión de genes relacionados con la resistencia, previamente identificados en nuestra cohorte de pacientes. También se analizó el perfil mutacional de las CTC utilizando el panel Human Comprehensive y, a continuación, se validaron las mutaciones en muestras de ctDNA mediante ddPCR. Además, se midió longitudinalmente la concentración de cfDNA para monitorizar tanto la respuesta al tratamiento como la progresión de la enfermedad. Por último, se comprobó si los PBMC podían ser un biomarcador pronóstico del riesgo de cáncer de mama y de la respuesta al tratamiento. Para ello, se recogieron muestras de sangre periférica de mujeres libres de cáncer y de pacientes con enfermedad temprana o avanzada antes del inicio de la terapia (visita 1). A continuación, se aislaron PBMC usando Lymphoprep y se analizó la expresión génica de un conjunto seleccionado de genes en mujeres libres de cáncer, con estadio temprano y avanzado de CM. En este último grupo, también se estudió la expresión génica entre pacientes con diferente respuesta terapéutica. Asimismo, se analizaron genes informativos sobre el riesgo de CM en pacientes con CM avanzado TN. Además, se realizó un co-cultivo paracrino con PBMCs naïve o de pacientes HR+ con una línea celular de cáncer HR+ para estudiar mediante RT-qPCR cómo la interacción entre ambos tipos celulares cambia su expresión génica.

Se incluyeron veinticinco pacientes con CM en estadio avanzado HR+/HER2- en el estudio para identificar biomarcadores en las CTCs. La edad media en el momento del diagnóstico de la enfermedad avanzada fue de 60 años. Todas las pacientes habían recibido CDK4/6i y ET como terapia de primera línea metastásica. Existe un sesgo hacia la administración de Palbociclib (68 % de las pacientes) en nuestra cohorte. Las pacientes no respondedoras (n=5) progresaron en un tiempo medio de 105 días, mientras que las pacientes respondedoras (n=20) progresaron en un tiempo medio de 601 días. Un total de 18 pacientes sufrieron progresión de la enfermedad, mientras que 10 fallecieron durante el seguimiento. El aislamiento de las CTCs

con RosetteSep evitó el sesgo introducido por los métodos basados en las propiedades moleculares. Con este método se enriquecieron todas las subpoblaciones de CTC y, en concordancia, se observó un fenotipo epitelial-mesenquimal con expresión de marcadores de células madre en las visitas 1, 2 y 3. Además, la enumeración de CTC mediante el sistema CellSearch® se realizó en 12 pacientes en la visita 1 y 2 mientras que en 7 pacientes en la visita 3, mostrando que los pacientes con ≥ 1 CTC en la visita 2 tenían una supervivencia más corta.

Además, tener ≥ 1 CTC asocia con una baja expresión de *ALDH1A1* en la visita 3, momento en el que este gen también asocia con el peor resultado. En cuanto a la expresión génica de la CTCs, en la visita 1 las pacientes no respondedoras tenían una mayor expresión de los genes *STAT3*, *PRKCB* y *MAPK3*, mientras que las pacientes respondedoras tenían una mayor expresión de los genes implicados en la vía *CCND1-CDK4/6*. Asimismo, la expresión elevada de *CCND1*, *DUSP5* y *GZMB* identifica a los pacientes que tendrán una respuesta más prolongada a la terapia combinada (>2 años). Además, la expresión de *DUSP5*, *CDK4* y *PALB2* asocia a un mejor pronóstico de las pacientes. En la visita 2, las pacientes que no respondieron tenían una mayor expresión de los genes *CDH1*, *CDKN1C* y *CUL1*, siendo *CDKN1C* también indicativo de un peor pronóstico. En la visita 3, *BAX*, *EZH2*, *PLAU*, *RELA*, *NFKBIα* y *SNAIL1* son informativos de la resistencia intrínseca y adquirida a CDK4/6i plus ET. Curiosamente, la expresión de *HDAC6* y *CDC7* fue específicamente informativa de resistencia adquirida, mientras que los niveles elevados de *CASP8* se encontraron en pacientes con resistencia intrínseca. Además, *ALDH1A1* y *STAT3* se asociaron con la peor supervivencia de los pacientes. No se encontró ninguna asociación entre la expresión génica de CTC y las características clínicas de las pacientes (terapia recibida, localizaciones metastásicas, etc.).

En la visita 1, se descubrió que la expresión combinada de $STAT3^{alto}PRCKB^{alto}$ -$CDK6^{bajo}$ determina qué pacientes responderán a la terapia combinada. Esta firma fue validada en una cohorte interna (n=12), mostrando la misma precisión en la clasificación de pacientes. Además, la firma también fue validada en una cohorte externa (n=16) en la que las CTCs fueron aisladas con Adnatest, que se basa en la expresión de marcadores de superficie (*EpCAM*, *HER2* y *EGFR*), lo que demuestra que esta firma clasifica a las pacientes independientemente del método de aislamiento o procedencia de las muestras.

Además, también se encontró una asociación entre la expresión génica de las CTCs. Así, en la visita 1 se identificó una asociación entre los marcadores mesenquimales *SNAIL1* y *VIM* así como entre los marcadores de células madre *ALDH1A1* y *PLS3*. También se encontró que la expresión de *PI3KCG* asociaba positivamente con *JAK2* y *CUL1* mientras que STAT3 con PRKCB. Asimismo, se encontró una asociación positiva entre *CDK4* y *6*. En la visita 2, se observó una asociación negativa entre *CDK4* y *CDKN1C*, mientras que una asociación positiva entre *CCND1* con *CDKN2C* y *STAT3* con *MAPK3*. Por último, en la visita 3 se identificó una asociación entre la expresión de *STAT3* con *PRKCB*, *JAK2* y *ALDH1A1* y dos genes apoptóticos, *BAX* y *CASP8*.

Se generó un modelo de línea celular resistente utilizando las líneas celulares hormonales MFC7 y T47D para estudiar la expresión de genes relacionados con la resistencia a CDK4/6i

en nuestra cohorte en estadio avanzado. La línea celular MCF7 se hizo resistente a Palbociclib (MCF7p-R) mientras que la T47D a Ribociclib (T47Dr-R). La adquisición de resistencia se confirmó mediante citometría de flujo, ensayos de proliferación y formación de colonias. El modelo MCF7p-R fue el mejor para estudiar los genes relacionados con la resistencia, ya que reflejaba con exactitud los patrones de expresión observados en las pacientes con CM en estadio avanzado.

Se realizó un análisis exhaustivo del ADN de las CTCs para identificar alteraciones relacionadas con la respuesta al tratamiento. Todos los pacientes compartían una mutación patogénica en *KMT2C* y *C/EBPα* en la primera visita. Curiosamente, todos los pacientes no respondedores incluidos presentaban mutaciones de pérdida de función en *ARID1A*, que promueve la progresión del ciclo celular junto con mutaciones en el gen *C/EBPα*. Asimismo, las pacientes respondedoras tenían una depleción en *ARID1B*, que evita la progresión del ciclo celular. También se encontró una deleción en el gen *HSP900A1* en el ADN de las CTC de los pacientes no respondedores. Dado que también se encontró en el ctDNA de todas las pacientes HR+/HER2- en estadio avanzado, se descartó como biomarcador de respuesta. Se realizó un estudio más profundo analizando el estado mutacional de *PI3K* en el ctDNA de pacientes que progresaron a CDK4/6i y ET, pero no se encontró ninguna asociación entre la presencia de mutaciones de PI3K y la respuesta a la terapia combinada o supervivencia de los pacientes. Sin embargo, se encontró una asociación entre tener *PI3K* mutado y alta expresión de *HDAC6*, así como *PI3K* wild-type y alta expresión de *CUL1*, *RAC2* y genes apoptóticos (*BAX* y *CASP8*). Estos resultados señalaron que la vía *PI3K* está alterada en pacientes con CM en estadio avanzado, que afecta a otras vías tumorigénicas. Además, el estudio longitudinal del cfDNA permite monitorizar la evolución de la enfermedad. Asimismo, la ratio de cfDNA mostró que las pacientes respondedoras tenían niveles de cfDNA más bajos que los no respondedores, lo que también se asociaba con la supervivencia de los pacientes.

Se incluyó a un total de noventa y ocho pacientes para estudiar el papel de los PBMC en el CM. Se incluyeron 20 mujeres libres de cáncer, 21 HR+ y 13 TNBC en estadio temprano, así como 26 HR+/HER2- y 18 TNBC en avanzado. Estudiando ambos subtipos, la edad media en el momento del diagnóstico del tumor primario (estadio temprano) fue de 59 años. Todas las pacientes fueron tratadas con quimioterapia. La edad media al diagnóstico de la enfermedad metastásica fue de 61 años. El subtipo HR+ fue tratado con CDK4/6i y ET y desarrollaron metástasis principalmente en hueso y vísceras. El subtipo TNBC se trató con quimioterapia y desarrolló metástasis principalmente en las vísceras. Los PBMC de pacientes con CM en estadio avanzado se aislaron con Lymphoprep antes del inicio de la terapia. Se observó una mayor expresión de *GZMB*, *KLF4* y *MYCL* en las pacientes respondedoras. A continuación, se analizaron los genes expresados diferencialmente en PBMC de mujeres libres de cáncer y en estadios iniciales de CM. *KITLG*, *KMT2D*, *KFL4* y *MYCL* se expresaron en mayor medida en las mujeres sin cáncer que en las pacientes con CM. Curiosamente, la expresión de *KITLG* disminuye progresivamente durante la enfermedad, probablemente debido a la transformación de las células malignas y a la alteración del microambiente mamario tumoral. Además, se observó la expresión de GZMB, una proteína liberada por las células inmunitarias efectoras,

tanto en la fracción enriquecida de CTC como en los PBMCs. Estos resultados también se estudiaron en el subtipo TN, en el que también se halló una mayor expresión de *KITLG*, *KLF4* y *MYCL* en las mujeres libres de cáncer.

Tras un co-cultivo paracrino, se estudió la expresión de los genes mencionados, comprobándose que la expresión de *KITLG* y *KLF4* disminuye significativamente en los PBMC naïve, pero no en los expuestas previamente al microambiente tumoral. También se observó que los niveles de leucocitos disminuían tras un ciclo de terapia, pero no todos los tipos de leucocitos disminuían por igual en todos los grupos de pacientes. Se calculó el cociente NLR como marcador de la respuesta inmunitaria. En el subtipo HR+, el NLR mostró unos niveles de estrés fisiológico normales tras la terapia de un ciclo, pero en el TNBC sigue habiendo pacientes con peores niveles de estrés fisiológico tras un ciclo de quimioterapia.

Considerando todos los resultados, se puede decir que nuestros datos refuerzan la utilidad clínica de utilizar las CTCs como marcadores pronósticos y predictivos para el manejo de pacientes con CM estadio avanzado y subtipo HR+/HER2-. Se demostró que la metodología de enriquecimiento negativo permite el aislamiento de diferentes subpoblaciones de CTCs, ya que se detectaron CTCs con fenotipo epitelial y mesenquimal en diferentes puntos temporales. No obstante, debe tenerse en cuenta la contaminación con linfocitos no eliminados en el análisis de CTCs. En nuestro caso, la expresión de *GZMB* se correlaciona con la respuesta terapéutica cuando se analizan las CTC, pero se trata de un gen liberado por efectores celulares inmunitarios.

Tras el análisis de las CTC, se confirma que el número de CTCs en sangre periférica permite determinar el pronóstico de las pacientes. Además, varios biomarcadores identificados en las CTCs podrían guiar la selección de la terapia en las visitas 1 y 3. Antes de iniciar la terapia, se ha descrito un aumento de la expresión de *STAT3*, *PRKCB* y *MAPK3* que promueve la resistencia a CDK4/6i y ET en la enfermedad avanzada. De hecho, ya se han desarrollado inhibidores de *STAT3* y *MAPK3*, cuya eficacia se está probando en combinación con CDK4/6i. *DUSP5* está altamente expresado en pacientes respondedores, inhibiendo la activación de *MAPK3*. Así pues, la expresión conjunta de ambos genes debe tenerse en cuenta antes de iniciar la terapia. Asimismo, también se observó que la respuesta más prolongada a la terapia combinada se produce en pacientes con alta expresión de genes implicados en el eje *CCND1-CDK4/6*. Es importante destacar que la firma desarrollada considerando la expresión de $STAT3^{alto}$-$PRCKB^{alto}$-$CDK6^{bajo}$ determina con precisión qué paciente con cáncer de mama estadio avanzado subtipo HR+/HER2 se beneficiará de la terapia combinada, independientemente del método de aislamiento de CTC utilizado. En la progresión de la enfermedad, se observó que varios mecanismos epigenéticos estaban alterados, como *EZH2* y *HDAC6*, de ahí que sería recomendable estudiar el perfil epigenético de las CTC. Se están probando inhibidores contra *EZH2* y *HDAC6* en varios tipos tumorales que tengan *ARID1A* mutado, una alteración identificada en pacientes no respondedores. De hecho, se ha estudiado la combinación de un inhibidor de *HDAC6* con inhibidores de *PI3K* o *CDK4*/6 como terapia combinada para pacientes con CM estadio avanzado subtipo HR+/HER2-. Además, la elevada

expresión de *CDC7* demostró que la progresión del ciclo celular no depende del eje CCND1-CDK4/6 en la adquisición de resistencia. Asimismo, es importante el papel dual de *CDK6*, ya que en la progresión de la enfermedad promueve el crecimiento tumoral a través de la interacción con los factores *NF-kB*. Evitando su interacción, se restablece la sensibilidad CDK4/6i. Algunos de los genes antes mencionados relacionados con la resistencia fueron validados sólo en la línea celular MCF7p-R, lo que pone de manifiesto la importancia de considerar las propiedades genéticas y físicas de los modelos seleccionados. Una de las terapias alternativas tras la resistencia a CDK4/6i son los inhibidores de PI3K. Se observó que las mutaciones de PI3K en el ctDNA de nuestra cohorte son frecuentes en las pacientes respondedoras y correlacionó con genes cuya expresión estaba alterada en las CTCs, como *CUL1*, que interacciona con *EZH2*, y *HDAC6*. Además, la alteración de los genes relacionados con apoptosis (*BAX* y *CASP8*) se encontró en la visita 3 y también correlacionó con la mutación de *PI3K*. Además del estudio del ctDNA, los niveles de cfDNA permitieron clasificar a las pacientes en función de la respuesta terapéutica a los 3 y 6 meses. Por último, el estudio del transcriptoma de PBMCs puede utilizarse para conocer la gravedad de la enfermedad. Por ejemplo, se produce una pérdida progresiva de *KITLG* a través de la transformación maligna y la expresión de *KLF4* se pierde en la enfermedad avanzada. Del mismo modo, estas células también informan sobre la respuesta a la terapia, ya que se encontró una baja expresión de *KLF4*, *MYCL* y *GZMB* en pacientes no respondedoras. De hecho, la expresión de *GZMB* se ha utilizado para predecir la respuesta al tratamiento en varios tipos de cáncer. Sin embargo, es necesario seguir caracterizando la expresión de cada tipo de leucocito y estudiar cómo cambian sus niveles en función del régimen terapéutico. Además, se confirmó que la interacción entre PBMC y las células tumorales afecta a la expresión de las células inmunitarias y, por tanto, a la respuesta inmunitaria.

Por lo tanto, teniendo en cuenta todos los resultados, se demuestra la necesidad de combinar la información transcripcional, mutacional y epigenética de las CTCs, así como los datos de otro material derivado del tumor para comprender la biología tumoral y guiar la terapia de los pacientes. En este sentido, nuestros resultados mostraron que el estudio de CTCs, cfDNA y PBMCs proporciona información clínicamente relevante para el manejo de pacientes con CM estadio avanzado y subtipo HR+/HER2- antes del inicio de la terapia o en la progresión de la enfermedad. Por lo tanto, se requiere una mayor caracterización molecular de este material circulante en una cohorte más grande y en ensayos clínicos para complementar la información clínica para conseguir una medicina de precisión.

SUMMARY

SUMMARY

Breast cancer (BC) is the leading cancer among women, being the metastatic stage the main cause of death. The incidence and mortality of this carcinoma increased in the last years for different biological and socio-cultural changes. It is described as a heterogeneous disease whose prognosis depends on its anatomic characteristics, the TNM score and the cancer subtype. Up to now, the BC is classified in three subtypes based on the expression of the human epidermal growth factor receptor 2 (HER2) and the expression of two hormonal receptors (HR): estrogen receptor (ER) and progesterone receptor (PR). The HR+ or luminal subtype is characterized for the expression of hormonal receptors. It is the most common and less aggressive subtype, being divided into luminal A or B considering the proliferation rate and the expression of HER2. The HER2+ pure subtype is characterized for the overexpression of HER2. Lastly, the triple negative (TN) subtype does not express the above-mentioned biomarkers. It is the most aggressive subtype with the worst outcome. The therapy regimen for the HR+ subtype is endocrine therapy (ET), for HER2 pure subtype is HER2-targeted therapy while for the TNBC subtype chemotherapy or immunotherapy.

Around 70 % of patients with BC are diagnosed with the HR+/HER2- subtype. Regardless of cancer stage (early or advanced), this subtype was traditionally treated with ET, but in advanced stages endocrine resistance is commonly acquired. Hence, the FDA approved the combination of cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) along with ET to treat HR+/HER2- stage IV BC patients as first-line metastatic setting. The CDK4/6i commercially available are Palbociclib, Ribociclib and Abemaciclib. This therapeutic approach enhances patients' survival for more than 2 years with barely secondary effects, as it was demonstrated in PALOMA, MONALEESA and MONARCH trials. Nevertheless, a total of 20 % of patients do not respond to this polytherapy due to intrinsic resistance. Moreover, those patients who initially respond ended up developing an acquired resistance. As the tumor biopsy can be performed years before disease recurrence, it could not reflect the current tumor characteristics. Besides, a biopsy of metastatic locations is not always feasible. Therefore, in order to determine which patients will benefit from the combination of CDK4/6i plus ET, the current tumor characteristics should be studied, being the liquid biopsy a great strategy.

Liquid biopsy is defining as the sampling and analysis of tumor-derived material in different body fluids such as blood, saliva and urine. This approach overcomes the tumor biopsy

disadvantages as allows studying the tumor in a repetitive, real-time and non-invasive way. Combining the information obtained from both biopsies, it will improve patients' management in terms of early diagnosis, prediction of therapy response, detection of resistance acquisition, identification of target therapies, etc. The tumor-derived material most study through liquid biopsy is the circulating tumor DNA (ctDNA), but recently it has been broadened to circulating tumor cells (CTCs), microRNAs (miRNAs), circulating tumor RNA (ctRNA) and extracellular vesicles (EVs).

The cell-free DNA (cfDNA) is released into the circulation by apoptotic tissue and hematological cells in physiological conditions. Its lifespan varies from 16 min to 2.5 hours. The ctDNA is a small fraction of cell-free DNA (< 1%) releases from primary tumor, metastasis or CTCs. The amount of ctDNA detected in the circulation depends on the number of cell deaths, tumor metabolisms, necrosis, apoptosis, etc. It is widely used in the clinic to track the mutational profile and to detect the minimal residual disease. However, CTCs are lately gaining importance as tumor-derived material in the oncology field. These cells are intact tumor cells that reflect the current tumor molecular characteristics. CTCs are released into the blood stream from primary tumor or metastatic sites both actively or passively, being able to form other metastatic locations. CTCs are extremely rare in the circulation, 1-10 cells as single cells or clusters per 10 mL of whole blood, being its lifespan between 1-2.4 hours. Their short lifespan along with their scarcity makes their isolation and characterization complicated. The main challenge in the oncology field is identified which CTC subpopulation initiates the metastatic lesion. For that, the isolation and detection methods should avoid the bias introduced when these processes are based on CTC marker expression or physical characteristics. Currently, it is approved the CTC enumeration by CellSearch® system as informative of patient's prognosis, but this approach does not inform about tumor characteristics, which are needed for decision making. Consequently, it is important the study of CTCs transcriptomics, proteomics and epigenomics to fully comprehend the current tumor genetic landscape. In addition, it is gaining importance the generation of models based on CTCs to study the processes related with cancer development, metastasis formation or testing drugs safety and efficacy, in order to get a precision medicine.

Regarding resistance acquisition in HR+/HER2- stage IV BC patients, several CDK4/6i resistance mechanisms have been proposed, being studied mainly in the ctDNA. As CDK4/6i and ET act in the same axis, certain resistance mechanisms are common, which complicates the study of polytherapy resistance. The resistant mechanism proposed are grouped as cell-cycle specific, such as loss of *RB1*, alteration of *CDK* intrinsic inhibitors, *CCND1* or *CDK4*/6 amplification, etc., as well as non-specific of cell cycle, such as the activation of signaling pathways (ER1 or PI3K/AKT/mTOR), aberrations in *FGFR*, epithelial-mesenchymal transition, etc. However, any resistance mechanism has been clinically validated so far.

All things consider, the current clinical oncological challenge is the management of HR+/HER2- stage IV BC patients resistant to polytherapy. In this context, the aim of this thesis is to identify biomarkers related with CDK4/6i plus ET response and resistance characterizing

the circulating material in HR+/HER2- BC patients. For that, it was collected blood from HR+/HER2- stage IV BC patients at different time points: when the metastatic disease was diagnosed (visit 1), after one-cycle therapy (visit 2), at disease progression (visit 3) and every 3 months since visit 1 up to 24 months. Considering the time of disease progression, patients were classified as *non-responders* or with intrinsic resistance if disease progression occurs within 6 months after visit 1; and as *responders* or with acquired resistance if disease progression occurs after 6 months from therapy initiation.

From the freshly-peripheral blood sample, CTCs were isolated with a negative enrichment method, the RosseteSep. Then, these cells were characterized at transcriptomic level using the PanCancer Pathway Panel (nCounter techonology) to discover the differential expressed genes between patients with different therapy response or resistance acquisition. In addition, an *in vitro* model resistant to CDK4/6i was develop to study the expression of resistant-related genes previously identified in our patient cohort. It was also analyzed the CTC mutational profile using the Human Comprehensive Panel and then, the mutations were validated in paired-ctDNA samples by ddPCR. Besides, the cfDNA concentration was measure longitudinally to monitor both therapy response and disease progression. Lastly, it was checked out if PBMCs could be a prognostic biomarker and predictive of therapy response. For that, peripheral blood samples from cancer-free women and patients with early or advanced disease were collected before therapy initiation (visit 1). Then, PBMCs were isolated using Lymphoprep and the gene expression of a selected set of genes was analyzed on cancer-free women, early and advance BC stage. In the last group, it was also studied the gene expression between patients with different therapy response. Likewise, prognostic genes were analyzed in TN stage IV BC patients. In addition, a paracrine co-culture assay with naïve or HR+ BC PBMCs was performed with a HR+ cancer cell line to study by RT-qPCR how the crosstalk between both cell types changes their gene expression.

A total of twenty-five HR+/HER2- stage IV BC patients were included to identify biomarkers in the CTCs. The mean age at diagnosis of advanced disease was 60 years. All have received CDK4/6i plus ET as a first-line metastatic therapy. There is a bias towards the administration of Palbociclib (68 % of patients) in our cohort. The *non-responder* patients (n=5) progressed in a mean time of 105 days, while the *responder* patients (n=20) progress in a mean time of 601 days. A total of 18 patients suffered from disease progressed, while 10 have died during the follow-up. The CTCs isolation with RosetteSep avoids the bias introduced by methods based on molecular properties. With this approach all CTC subpopulations were enriched, and in agreement an epithelial-mesenchymal phenotype with the expression of stem-cell markers was observed at visit 1, 2 and 3. In addition, the enumeration of CTCs by CellSearch® system was available in 12 patients at visit 1 and 2 while in 7 patients at visit 3, showing that patients with ≥ 1 CTC at visit 2 had shorter survival. Moreover, having ≥ 1 CTC associated with low *ALDH1A1* expression at visit 3, time point in which this gene also associated with worst outcome. Concerning the CTC gene expression, at visit 1 *non-responder* patients had higher expression of *STAT3*, *PRKCB* and *MAPK3* genes, while *responder* patients had a higher expression of genes involved in the CCND1-CDK4/6 pathway. Likewise, high

expression of *CCND1*, *DUSP5* and *GZMB* identified patients that will have a longer response to polytherapy (>2 years). Moreover, *DUSP5*, *CDK4* and *PALB2* expression associated with better patient's prognosis. At visit 2, *non-responder* patients had higher expression of *CDH1*, *CDKN1C* and *CUL1* genes, being *CDKN1C* also informative of worse patient's prognosis. At visit 3, *BAX, EZH2, PLAU, RELA, NFKBIα* and *SNAIL1* are informative of CDK4/6i plus ET both intrinsic and acquired resistance. Interestingly, *HDAC6* and *CDC7* expression were specifically informative of acquired resistance, while high levels of *CASP8* were found in patients with intrinsic resistance. Moreover, *ALDH1A1* and *STAT3* associated with worst patient's survival. No association was found between CTC gene expression and patient's clinical characteristics (therapy received, metastatic locations, etc.).

At visit 1, it was found that combining the expression of $STAT3^{high}PRCKB^{high}$ $CDK6^{low}$, it is likely to determine which patients will response to polytherapy. This signature was validated in an internal cohort (n=12), showing the same accuracy classifying patients. In addition, the signature was also validated in an external cohort (n=16) in which CTCs were isolated with Adnatest, which is based on the expression of surface markers (*EpCAM*, *HER2* and *EGFR*), which demonstrate that this signature classifies patients regardless the isolation method or samples procedence.

In addition, it was also found an association between CTC gene expressions. Thus, at visit 1 it was identified an association between the mesenchymal markers *SNAIL1* and *VIM* as well as between the stem cell markers *ALDH1A1* and *PLS3*. It was also found that *PI3KCG* expression positive associated with *JAK2* and *CUL1* while *STAT3* with *PRKCB*. Likewise, it was found a positive association between *CDK4* and *6*. At visit 2, it was observed a negative association between *CDK4* and *CDKN1C*, while a positive association between *CCND1* with *CDKN2C* and *STAT3* with *MAPK3*. Lastly, at visit 3 it was identified an association between the expression of *STAT3* with *PRKCB*, *JAK2* and *ALDH1A1* and two apoptotic genes, *BAX* and *CASP8*.

A resistant cell line model was generated using the hormonal cell lines MFC7 and T47D to study the expression of genes related with CDK4/6i resistance in our stage IV cohort. MCF7 cell line was resistant to Palbociclib (MCF7p-R) while T47D resistant to Ribociclib (T47Dr-R). The resistance acquisition was confirmed by flow cytometer, proliferation and colony formation assays. The MCF7p-R model was the best for studying resistant-related genes, as it accurately mirrored the expression patterns observed in the HR+/HER2- stage IV BC patients.

A comprehensive DNA-seq analysis on CTCs was performed to identify alterations related with therapy response. All patients shared a pathogenic mutation in *KMT2C* and *C/EBPα* at visit 1. Interestingly, all *non-responder* patients included had a loss of function *ARID1A* mutations, which promotes cell cycle progression along with mutations in *C/EBPα* gene. Likewise, the *responder* patients had a depletion in *ARID1B*, which avoids cell cycle progression. A deletion in *HSP900A1* gene was also found in the CTCs' DNA from *non-responder* patients. Since it was also found in the ctDNA from all HR+/HER2- stage IV patients, it was discarded as a response biomarker. A deeper study was done analyzing the *PI3K*

mutational status in the ctDNA from patients who progress on CDK4/6i plus ET, but no association was found between the presence of *PI3K* mutations and polytherapy response or patient's outcome. However, it was found an association between having *PI3K* altered and high expression of *HDAC6*, as well as *PI3K* wild-type and high expression of *CUL1*, *RAC2* and apoptotic genes (*BAX* and *CASP8*). These results pointed out that the *PI3K* pathway is altered in stage IV BC patients and affect other tumorigenic pathways. In addition, the cfDNA longitudinally study let's monitor the disease evolution. Likewise, a cell-DNA ratio showed that *responder* patients had lower cfDNA levels than *non-responders*, which also associated with patients' survival.

A total of ninety-eight patients were included to study the role of PBMCs in BC. It was included 20 cancer-free women, 21 HR+ and 13 TNBC stage I-III as well as 26 HR+/HER2- and 18 TNBC stage IV BC patients. Studying both subtypes, the mean age at diagnose of primary tumor (stage I-III BC) was 59 years. All patients were treated with chemotherapy. The mean age at diagnosis of metastatic disease was 61 years. The HR+ subtype was treated with CDK4/6i plus ET and develop metastasis mainly in bone and viscera. The TNBC subtype was treated with chemotherapy and develop metastasis mainly in the viscera. The PBMCs from stage IV BC patients were isolated with Lymphoprep before therapy initiation. Higher expression of *GZMB*, *KLF4* and *MYCL* was found in *responder* patients. Then, the differential expressed genes were analyzed in PBMCs from cancer-free women and early-BC stages. *KITLG*, *KMT2D*, *KFL4* and *MYCL* were highly expressed in cancer-free women compare with BC patients. Interestingly, *KITLG* expression progressively decrease during disease probably due to malignant cells transformation and alteration of tumor breast microenvironment. In addition, the expression of *GZMB*, a protein released by effector immune cells, was found both in the enriched-CTC fraction and PBMCs. These results were also studied in the TN subtype, in which a higher expression of *KITLG*, *KLF4* and *MYCL* was also found in cancer-free women.

After a paracrine co culture, the expression of the above-mentioned genes was studied, finding that *KITLG* and *KLF4* expression significantly decreased in naïve PBMCs, but not in those previously exposed to cancer microenvironment. It was also observed that the leukocyte levels decreased after one-cycle therapy, but not all leukocytes types decreased equally in all patient groups. The NLR ratio was calculated as a marker of immune response. In the HR+ subtype, the NLR showed a normal physiologic stress levels after one-cycle therapy, but in the TNBC there are still patients with worse physiologic stress levels after one-cycle of chemotherapy.

Considering all the results, it can be said that our data reinforces the clinically utility of using the CTCs as prognostic and predictive markers for the management of HR+/HER2- stage IV BC patients. It was proved that the negative enrich methodology allows the isolation of different CTCs subpopulations, since it was detected CTCs with epithelial and mesenchymal phenotype at different time points. Nevertheless, it should be considered the contamination with no-depleted lymphocytes in the CTC analysis. In our case, the expression of *GZMB* correlates

with therapy response when CTCs are analyzed, but it is a gene released by immune cell effector.

After the CTC analysis, it is confirmed that the number of CTCs in the peripheral blood allows the determination of patient's prognosis. Additionally, several biomarkers identified in the CTCs might guide-therapy selection at visit 1 and 3. Before therapy initiation, the enhance expression of *STAT3, PRKCB* and *MAPK3* is described that promotes CDK4/6i plus ET resistance in advanced disease. In fact, it has already been developed inhibitors again *STAT3* and *MAPK3*, whose efficacy is being tested in combination with CDK4/6i. *DUSP5* is highly expressed in responder patients, inhibiting the *MAPK3* activation. Thus, the expression of both genes together had to be considered before therapy initiation. Likewise, it was also observed that the longest response to polytherapy occurs in patients with high expression of genes involved in the CCND1-CDK4/6 axis. Importantly, the signature developed considering the expression of $STAT3^{high}PRCKB^{high}CDK6^{low}$ accurately determine which HR+/HER2- stage IV BC patient will benefit from the polytherapy, regardless of the CTC isolation method used. At disease progression, it was observed that several epigenetic mechanisms were altered, such as *EZH2* and *HDAC6*, hence the epigenetic profile of CTCs should be studied. Inhibitors against *EZH2* and *HDAC6* are being tested in several cancers with *ARID1A*-mutated, an alteration identified in *non-responder* patients. In fact, it has been studied the combination of *HDAC6* inhibitor with *PI3K* or *CDK4/6* inhibitors as novel polytherapy for HR+/HER2- stage IV BC patients. Apart from that, the high expression of *CDC7* showed that the cell cycle progression does not depend on the *CCND1-CDK4/6* axis at resistant acquisition. Likewise, it is important the dual role of *CDK6*, since at disease progression promotes tumor growth through the interaction with *NF-kB* factors. Avoiding their interaction, it is restored the CDK4/6i sensitivity. Some of the above-mentioned resistant-related genes were validated only in the MCF7p-R cell line, what highlight the importance of considering the genetic and physical properties of the models selected. One of the alternative therapies after CDK4/6i resistance is PI3K inhibitors. It was found that *PI3K* mutations in the ctDNA from our cohort was common in *responder* patients and correlate with genes which expression changes in CTCs through the disease, such as *CUL1*, that interacts with *EZH2*, and *HDAC6*. Moreover, alteration of apoptosis-related genes (*BAX* and *CASP8*) was found at disease progression and also correlated with *PI3K* mutation. Apart from the study of ctDNA, the cfDNA levels could classified patients according to therapy response at 3 and 6 months. Lastly, the study of PBMCs transcriptome can be used to know the disease severity. For example, a progressively loss of *KITLG* occurs through malignant transformation and *KLF4* expression is lost in advanced disease. Likewise, these cells also inform about therapy response since low expression of *KLF4*, *MYCL* and *GZMB* was found in *non-responder* patients. In fact, *GZMB* expression is been used to predict therapy respond in various cancer types. However, it is necessary further characterization of the expression of each leukocyte type and study how their levels change due to the therapy regimen. In addition, it was confirmed that the interaction between PBMCs and cancer cells affect the expression of immune cells, and hence, the immune response.

Therefore, considering all results, it is proved the necessity of combining transcriptional, mutational an epigenetic CTC information, as well as the data from other tumor-derived material to understand tumor biology and tailor-patient therapy. In this sense, our results showed that the study of CTCs, cfDNA and PBMCs provide clinically relevant information to manage HR+/HER2- stage IV BC patients before therapy initiation or at disease progression. Therefore, further molecular characterization of these circulating material in a bigger cohort and in clinical essays is required to complement the clinical information to get a precision medicine.

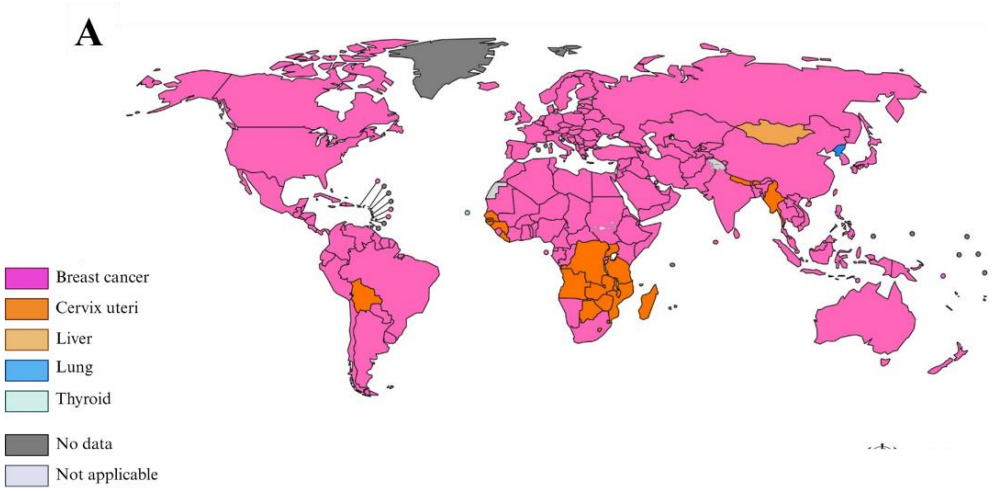
INTRODUCTION

INTRODUCTION

1. BREAST CANCER

1.1. Epidemiology

Breast cancer (BC) is the most common cancer among women worldwide[1,2], with an estimation of 2.3 million of new cases (11.7 %) in 2020[2]. In fifth position, this disease caused 6.9 % of cancer deaths[2]. Currently, the BC incidence is higher in developed countries (Figure 1A), while the mortality is higher in developing countries, likely to the lack of mammography screenings (Figure 1B). The upturned incidence and prevalence of BC are due to reproductive and hormonal changes such as early age of menstruation, later age of menopause, late motherhood, fewer births and/or oral contraceptives. Likewise, the lifestyle factors such as alcohol intake, obesity and reduced physical activity as well as, aging and overpopulation also increase the incidence and prevalence rates[2]. In the last few years, BC incidence has increased in developing countries as a consequence of social, economic and cultural changes. It is expected an increase of 28.4 million cases (47 %) in 2040, mainly in developing countries due to globalization and economic expansion[2].



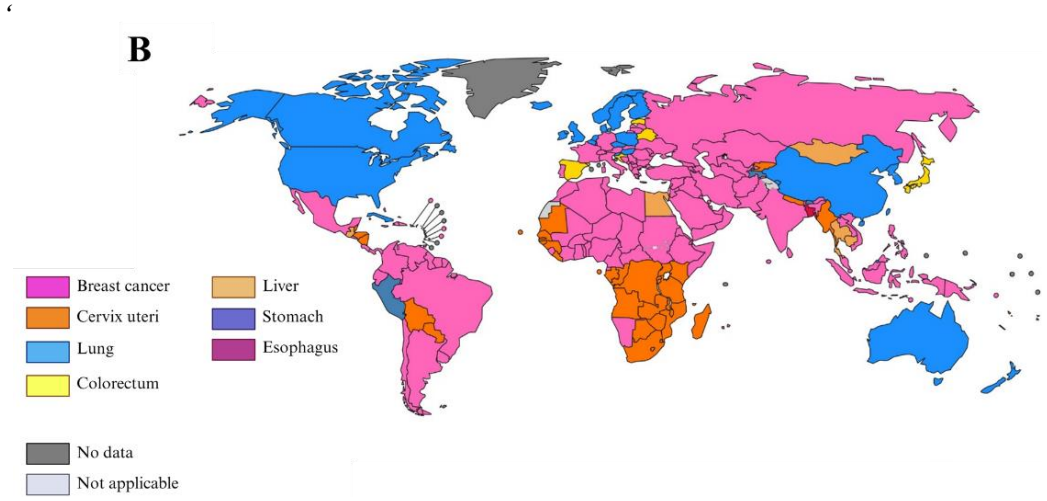


Figure 1. Ranking of cancer incidence and mortality among women in 2020. A) Cancer incidence, B) Cancer mortality. (Source GLOBOCAN 2020)

1.2. Breast cancer diagnosis and classification

BC is diagnosed through breast exam, imaging studies (mammogram, breast ultrasound, magnetic resonance imaging, etc.) and tissue biopsy or needle aspiration[3,4]. Following confirmation of BC diagnosis, it is determined the BC stage and the anatomic characteristics running different tests such as blood tests, bone scans, CT scans and/or PET scan[3,4]. Then, the BC subtype is determined considering the histopathological expression of tumor biomarkers[3,4]. The next step is surgical resection of the tumor (lumpectomy) or the entire breast (mastectomy). The systemic therapy is administered before surgery for tumor size reduction and after surgery to reduce the risk of cancer recurrence[5,6].

Breast carcinoma can be classified as ductal carcinoma, when tumor occurs on the surface of milk ducts, or lobular carcinoma when tumor occurs in the milk glands[4,6,7]. The prognosis of BC depends on tumor grade, biomarker expression and anatomic characteristics.

Firstly, the tumor grade describes the abnormality of breast cancer cells under the microscope. The classification is grade 1 if cancer cells look normal and have low proliferation rate, grade 2 if cancer cells do not look completely normal and proliferate faster than normal cells, while grade 3 if cells are abnormal and proliferate and spread quickly[4,8]. Then, higher-grade cancers proliferate and spread faster than lower-grade cancers[4].

Secondly, BC has been classified into three subtypes based on biomarker expression[5,9]. The main cancer biomarkers studied are human epidermal growth factor receptor 2 (HER2), and hormonal receptors (HR) such as estrogen receptor (ER) and progesterone receptor (PR) [6,9] (Figure 2).

- HR+/HER2- or Luminal subtype: 70 % of patients characterized for the expression of hormone receptors (HR+) such as ER and/or PR. It can be divided in Luminal A (40 %) or B (20 %) considering *KI67* and *HER2* expression[10]. The standard treatment is

endocrine therapy (ET)[11] such as Tamoxifen (blocks estrogen receptor), Aromatase inhibitors (AI) (inhibits the conversion of androgens to estrogen) and Fulvestrant (downregulation of ER)[5,9,12]. Those patients who express HER2 receptor can also receive HER2 target therapies[5,9]

- HER2+ subtype: A 10-15 % of patients characterized for the overexpression of HER2. These patients are treated with HER2+ target therapies or small-molecule inhibitor therapies [5,7,13].
- Triple negative (TN) subtype: A 15-20 % of patients do not express any of the above-mentioned biomarkers. It is the most aggressive subtype with the worst outcome. As there is no-associated target therapy, TNBC patients receive chemotherapy or immunotherapy[5,7,13].

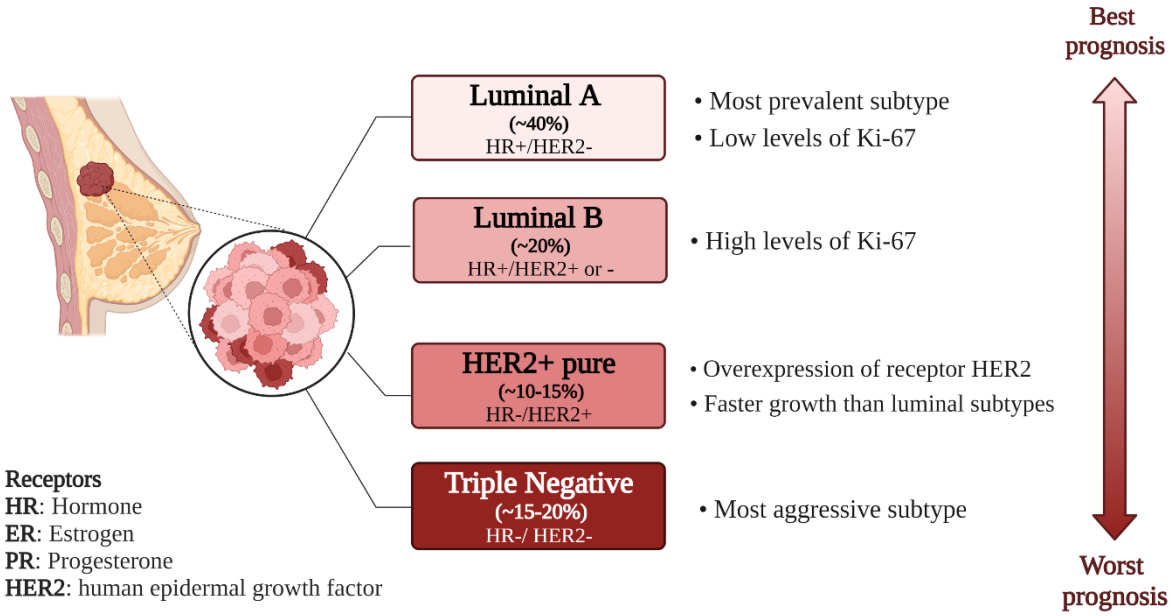


Figure 2. Characteristics of breast cancer subtypes. The luminal or hormonal subtype (HR) is the most common and the one with the best prognosis. HER2+ subtype is characterized for the overexpression of HER2. Triple negative breast cancer is the most aggressive with the worst prognosis.

Lastly, the anatomic characteristics are tumor size (T), lymph node status (N) and distant metastasis (M). This is known as TNM score. Considering this score, BC is ranged in different stages[4,6]:

- Stage 0 or non-invasive breast cancer. There is a mass called primary tumor (T1-4). There is no invasion of surround tissue, lymph nodes (N0) or distant tissues (M0) [6].
- Stage I-III or local metastasis/disease: Tumor can have any size (T1-4). The invasion spread to surrounding breast tissue or lymph nodes (N 1-3). There is no spread to distant sites (M0) [6].
- Stage IV or advanced breast cancer. Tumor can have any size (T1-4). Cells spread to axillary lymph nodes or distant sites forming secondary tumors

(M1). The most frequent sites where BC metastasizes are bone, lungs, liver, brain and distant lymph nodes [6].

Regarding the advanced disease, TNBC is more likely to recur compared with the other subtypes [5,14]. In 5 years, 85 % of TNBC patients do not progress compared with 99 % and 94 % for HR+ and HER2 pure subtypes, respectively [5,14]. The metastatic location differs among subtypes: HR+ subtype preferentially metastasize to bone, HER2+ subtype to the liver and TNBC to brain and lung[10,15–17]. The mean overall survival for TNBC is 1 year while 5 years for the other subtypes [5,6,15].

The metastatic disease is responsible for 90 % of BC related-deaths. This process stars when cells from malignant tumors spread to other parts in a sequential process, which is regulated by the epithelial-mesenchymal program (EMT) (Figure 3) [18,19]. First, cells from primary tumor start growing uncontrolled and new vessels are formed in the surrounding tissues. Then, tumor cells intravasate into the bloodstream and lymphatic vessels, where can form aggregates with hematological and lymphatic cells. If the disseminated cells overcome the immune destruction, growth suppressors, genome instability, etc., the cells might extravasate to distant locations forming micrometastasis. Finally, initiator metastatic cells proliferate, establish vascularization and resist host immune response until the macrometastasis formation[9,18,19]. This shedding of circulating tumor cells (CTCs) in the blood system is common, but only the initiator metastatic clones complete the steps required for the metastatic cascade. Nevertheless, finding CTCs in the bloodstream does not prove that metastasis has occurred [18].

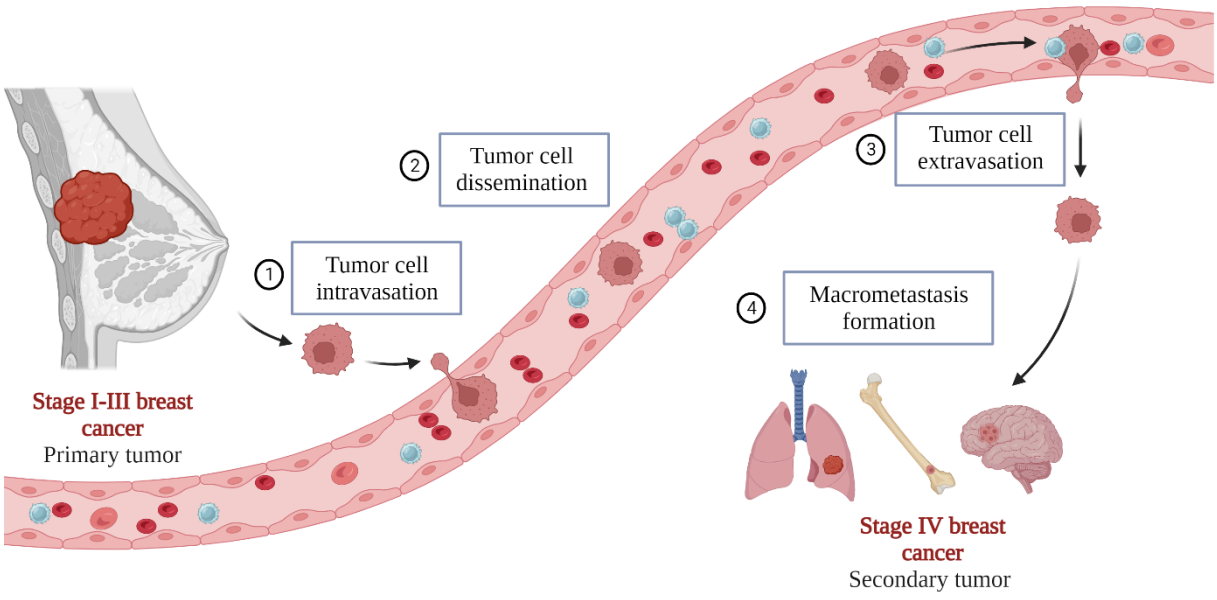


Figure 3. Metastatic cascade. Tumor cells are shed from the primary tumor and intravasate to the peripheral blood system (1). Then, cells are disseminated as single cells or clusters (2). If tumor cells survive in the blood system, they can extravasate in distant sites, forming secondary tumors (3-4).

2. LIQUID BIOPSY

Tumors are genetically heterogeneous and dynamic units, whose molecular profile evolves spatially and temporarily. Then, it is important to have prognostic and predictive tumor markers to guide cancer management during the disease [1,20,21]. Primary tumor biopsy is the gold-standard method for tumor analysis. Nevertheless, this technique has several drawbacks: invasiveness, impossibility of serial testing, no representation of tumor heterogeneity, no real time information, high procedures costs, etc[21,22]. Metastatic biopsies have the same disadvantages, but also the inaccessibility in many cases[11,20]. Liquid biopsy overcomes the above-mentioned problems by sampling body fluids such as blood, saliva or urine to study tumor-derived material in real-time and many times as needed with a non-invasive approach (Figure 4)[21,23]. The tumor-derived materials released into de peripheral blood that can be analyzed by liquid biopsy are the circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free RNA, tumor-derived proteins, extracellular vesicles (EVs) and tumor-educated platelets[1,11,21]. These tumor components can be used for different purposes such as early detection of minimal disease, monitor therapy response, therapy selection etc[1,21]. Likewise, liquid biopsy can be also used to study cells from the tumor microenvironment[24,25].

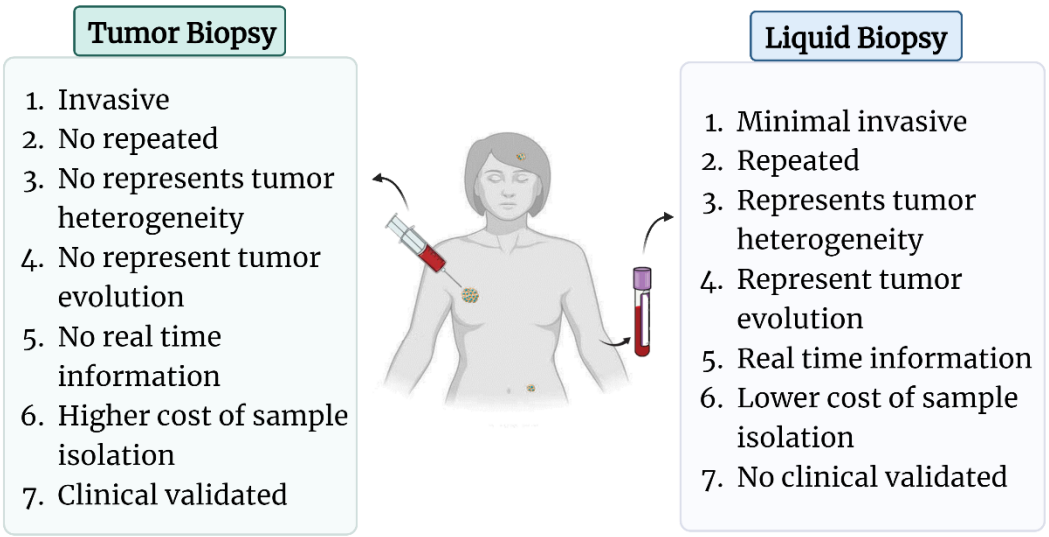


Figure 4. Comparison of tumor and liquid biopsy. Tumor biopsy is the gold standard method to characterize primary or secondary tumors but it does not represent the current tumor molecular profile due to spatial and temporal heterogeneity. Liquid biopsy is a new approach that monitors the tumor in real-time in a non-invasive and repeated approach.

In the BC metastatic setting, the liquid biopsy has a special interest since metastasis may occur years after primary tumor resection[26]. At that point, primary tumor biopsy might not provide information on the current tumor molecular characteristics[27,28].

2.1. Circulating tumor cells (CTCs)

There are several CTCs subpopulations that differs in genetic signatures, proliferation rates, aggressiveness and/or drug sensitivity due to spatial and temporal tumor

heterogeneity[11,29]. These cells are shed from primary tumor and/or metastasis actively (via EMT) or passively (detached as single cells or clusters)[11,29,30]. It is a rare cell fraction in the peripheral bloodstream, one to ten CTCs per million of white blood cells and per billion of red blood cells[29,31–33], with a lifespan of 1-2.4 hours[28,33,34]. In the circulation, these circulating cells exist as single cells and as circulating tumor microemboli or CTC clusters, which are groups of ≥ 2 CTCs or CTCs with other circulating cells. It is described that clusters have greater metastatic potential, resistance to blood shear forces, anoikis, immune attack and apoptosis[11,34].

CTCs represent pure and intact tumor cells, providing an opportunity to determine molecular and functional tumor characteristics. The latter cannot be achieved with any other tumor-derived material. Currently, there are several methods to isolate CTCs considering cell properties. Consequently, depending on the method selected, different CTC subpopulations are enriched.

2.1.1. CTCs isolation based on biological properties

These isolation methods are also known as protein expression-based technologies, since identify specific biomarkers from CTCs surface using antibodies[33]. The first-FDA validated platform to isolate CTCs was the **The CellSearch® system** (Menarini Silicon Biosystems) [35,36]. It enriches immunomagnetically CTCs that express the Epithelial Cell Adhesion Molecule (EPCAM+), which are then identified with antibodies anti-cytokeratins (CK8, 18, 19), anti-CD45 and DAPI for nucleus staining[35,36]. This technology ignores subpopulations with mesenchymal or stem-cell phenotypes[11]. It has been validated the association between CTC enumeration by CellSearch and patient's prognosis. Thus, ≥ 5 CTCs per 7.5 mL of blood is associated with poor prognostic in stage IV breast and prostate cancer, while ≥ 3 CTCs in colorectal cancer [11,37].

Other non-validated EpCAM strategies are the microfluidic devices CTC-Chip, NanoVelco system and GmbH cell collector that captures CTC EpCAM+ from peripheral arm vein[37]. In addition, to overcome EpCAM expression bias, other markers are considered such as MUC1 or EGFR. **The AdnaTest ™** (QIAGEN) enriches CTCs using immunomagnetic beads coated with antibodies against surface markers. The captured cells are detected by PCR-based methods, analyzing the expression of *HER2*, *MUC1* and *EpCAM* [38,39]. Another option is to deplete CD45+ cells [37]. Dynabeads® (ThermoFisher Scientific) and EasySep™ (StemCell Technologies) use immunomagnetic beads that recognize surface markers to remove unwanted cells or enrich CTCs, respectively [40]. Lastly, the **RosetteSep ™** (StemCell ™ Technologies) uses a cocktail of different antibodies to recognize different blood cell populations, which will be later removed by density gradient centrifugation[40].

2.1.2. CTCs isolation based on physical properties

These technologies identified CTCs considering physical properties such as cell size, deformability, electric charge, etc. **Parsortix® Cell Separation System** (ANGLE North America, Inc.) is a microfluidic-based technology validated by the FDA for BC management.

This system captures and harvest cells from body fluids based on cell size and deformability, since it is described that tumor cells are larger and less deformable than hematopoietic cells[33,41]. This technology isolates both epithelial and mesenchymal phenotype[41,42]. Other technologies are the ISET® system (Rarecells diagnostic) and Metacell® technology (Biovendor), filtration methods based on cell size, although do not enrich smaller CTCs[37]. DEP-based platforms such as DEPArray™ (Menarini Silicon Biosystems) and the ApoStream™ (Precision for Medicine, Inc) separate cells considering the cell capacitance and conductivity, letting differentiate CTCs from blood cells. Lastly, Screen Cell® (ScreenCell) uses micro-filters to separate the larger CTCs from leukocytes[40].

After the isolation, viable CTCs can be detected with functional assays. The EPISPOT assay (Epithelial Immunospot) detects proteins released or shed by CTCs after short-term cultures in a membrane coated with antibodies and after removing leukocytes via CD45 depletion[32,37,43]. The CAM assay (Vita-Assay) determines CTC invasiveness by measuring the uptake of CAM proteins by CTCs after digesting the adhesion matrix [32,37]. TelomeScan (Oncolys BioPharma), measures the telomerase activity in CTCs with adenoviruses, which marks cancer cells with green fluorescence protein [32,37]. In addition, a downstream analysis is needed for CTC detection and characterization[32,37]. For that, different approaches can be considered. The RT-qPCR quantifies RNA sequences with low starting material, although fails in unpurified samples[32,37,44,45]. Next Generation Sequencing (NGS) is a costly multiplex approach that detects a large number of genetic alterations, but the DNA concentration, length and quality is challenging[32,37,46,47]. In situ hybridization (FISH) identifies genome amplifications while immunofluorescence techniques (IF) detect protein markers [32,37]. Single-cell level analysis is being used to determine the real clinical value of CTCs, but novel algorithms are needed to handle technical noise, expression variability, etc[20,48].

Lately, it is gaining importance the generation of BC models using CTCs to study the characteristics of the original tumor[49]. For example, Koch *et al*. generated a CTC derived cell line from a HR+/HER2- stage IV BC patient. The copy number alteration profile was similar between the cell line and the CTCs from the blood system[49]. Besides, the cell line was sensitive to Palbociclib, then, it could be used to guide-therapy selection [49]. Next, using the CTC-derived cell line, the group generated a PDX mouse model in which the primary tumor and metastasis mirrored the patients' tumor characteristics: ductal primary tumor and bone metastasis[49]. In other study carried out in our group, CTCs from stage IV BC patients were isolated using RosetteSep and were cultured in hypoxic conditions using nanoemulsions to support the CTC growth[50]. Those CTCs with mesenchymal and steam features were able to growth *ex vivo*. The culture time did not depend on the number of CTCs enumerated by CellSearch, although it associated with patient's outcome[50]. Kolostova *et al*. isolated CTCs from prostate, gastric and ovarian cancers using the MetaCell technology[51–53]. These cells kept viable for 14-28 days both in the membrane filter on a culture plate or directly culture on a plate. It was observed that under this culture conditions CTCs were larger, paler and more elongate. On the membrane, CTCs had an epithelial phenotype, while those cells that growth directly on the plate showed

greater plasticity and invasiveness. Lastly, this group analyzed chemo-resistance-related genes on the CTCs cultured to determine therapy effectivity [32,53].

Despite the technological advances, the platforms are costly and not sensitive enough considering the low number of CTCs, the contamination with blood cells, the lack of a common marker in CTCs surface and molecular changes during the disease[31–33,35,36]. Besides, CTCs are fragile and easily destroyed during the isolation, enrichment, detachment from different devices and culture[36]. Leukapheresis, a procedure to isolate the white blood cells from the blood, has been proposed to capture a higher number of CTCs due to sampling higher blood volumes, overcoming some of the above-mentioned limitations. However, it comes with technical hindrances such as no-standardized protocols, stuffy pores due to the high number of leukocytes and patient care challenges [54].

2.2. Circulating tumor DNA (ctDNA)

The cell-free DNA (cfDNA) is released by apoptotic tissue and hematological cells in physiological conditions as infectious or inflammatory disease, diabetes, cancer, myocardial infarction, during exercise, pregnancy, etc.[20,55]. Its length is around 143 to 180 bp, having a lifespan from 16 min to 2.5 hours in the bloodstream[23,56]. The circulating tumor DNA (ctDNA) is the fraction of cfDNA (< 1 %) released from the primary tumor, metastasis or CTCs both passively (necrosis or apoptosis) and actively (vesicles)[11,20,35,37]. The only approach to differentiate ctDNA from cfDNA is sequencing tumor mutations[35,47]. The ctDNA represents not only the genomic landscape but also the intratumor clonal heterogeneity[35,57]. Its function is not known, but it is described a possible role in macrophage accumulation, inflammation after cell necrosis or as a communication system[57–59]. The amount of ctDNA detected by liquid biopsy depends on the number of cell deaths, tumor metabolism, proliferation rate, vascularization and tumor location [11]. It has been studied its use to diagnose cancer, detect residual tumors and metastases, but mainly for identifying or monitoring mutations, mainly those that associated with therapy efficacy[11,35,60–63]. This tumor-derived material is easily isolated based on centrifugation, immunomagnetic beads, silica column-based enrichment, phenol-chloroform-based extraction, vacuum generation, etc [35], but these isolation methods do not discriminate between cfDNA and ctDNA[64]. Then, NGS, digital droplet PCR (ddPCR) reaction, BEAMing assay, mass-spectrometry or hypermethylation analysis are needed[20,62]. NGS detects a large number of genetic alterations, but it is limited to ctDNA length and quality [11,20,35]. Concerning ddPCR and BEAMing assay, are costly, rapid, sensitive and precise technologies that analyses a small number of well-known mutations with little input material [11,20,35]. The mass-spectrometry analysis is under validation, but it seems to be sensitive and specific with low input material[35,65]. The ctDNA methylation is determined by methylation-specific PCR, which assesses the methylation status of one or two CpG sites at a time,[35,57,66,67] and methylation arrays, that are quite expensive[68,69]. The longitudinal monitorization of cfDNA and ctDNA levels is used as a prognostic biomarker to determine therapy response, while ctDNA mutational characterization is a useful biomarker to select targeted therapies [27,70 71].

The FDA approved three tests related to ctDNA analysis. Firstly, **EGFR mutation test visit 2** (Roche Diagnostics), a RT-PCR test that determines *EGFR* mutational status in cfDNA from non-small cell lung cancer patients at diagnose[37,72]. Secondly, **EpiproColon®** VISIT 2.0 CE kit (Epigenomics AG), a RT-PCR assay to determine qualitatively the methylation pattern of the *SEPT9* gene in plasma from colorectal cancer patients. Lastly, **Therascreen *PIK3CA* RGQ PCR Kit** (QIAGEN), a RT-PCR test that detects 11 mutations in the *PI3KCA* gene to determine which BC patients are eligible for receiving Alpelisib in combination with Fulvestrant[73,74]. Currently, the FDA is reviewing the **CancerSEEK** device (Agena Bioscience), which analyses circulating proteins and ctDNA by mass spectrometry for detecting early-stage cancers in asymptomatic patients older than 65 years. Besides, this test also determines in which organ the cancer began[35,75]. There are other platforms under development for minimal residual disease detection in the ctDNA. Firstly, the Galleri test (GRAIL) is based on ctDNA methylation for cancer detection[76]. Secondly, RaDaR (Inivata) analyses 48 tumor-specific variants in the ctDNA from lung, breast, colon and head and neck squamous cell carcinoma patients to detect minimal residual disease[60,61,77].

The ctDNA can be studied as a prognostic and predictive biomarker, although to compare the results from different studies is needed the establishment of timepoints for sample collection, protocols standardization and use the same thresholds[27, 37].

2.3. Other tumor-derived material

Cancer cells release extracellular vesicles (EVs) for intercellular communication[78,79]. These vesicles have an endosomal or plasma membrane origin, called exosomes (50-100 nm) and microvesicles (50 nm -10 μm), respectively[80]. Both EVs contain a wide variety of biomolecules such as RNA, lipids, proteins and DNA that promotes tumorigenesis, angiogenesis, invasion and metastasis formation[78,79]. Likewise, it is described that might transmits drug resistance through functional proteins and micro-RNAs[81]. Commonly, EVs are isolated from the supernatants of cultured cells by ultracentrifugation or kits such as miRCURY exosome (QIAGEN), ExoQuick® (System Bioscience) and Total Exosome Isolation Reagent (Invitrogen)[82].

Little is known about circulating tumor RNA (ctRNA) such as miRNA or long-non coding RNAs[83]. ctRNA is released actively within EVs or passively by cell apoptosis or necrosis[84–87]. Currently, it is used as a complementary biomarker to determine the expression of cancer related signatures that reflect the tumor microenvironment, patients' prognosis and therapy efficacy. The detection and identification of ctRNA can be done by microarray assays, RT-qPCR and NGS[83,86,87]. However, standardized protocols to isolate, stabilized and selected specific ctRNA are required. Concerning circulating tumor proteins, are used as prognosis and predictive markers in cancer[88]. These proteins are secreted or shed by cancer and immune cells into body fluids[89]. Changes in protein levels promote proliferation, invasion and are used to track disease progression[90,91]. Proteins can be detected via ELISA, mass spectrometry, antibody array and aptamer based proteomic etc, but a higher sensitivity is needed[88].

Platelets are small blood cells released from the bone marrow, being its main role stops hemorrhages[92]. It is well-described that tumor-educated platelets promote tumor metastasis through various processes. Firstly, promoting angiogenesis in order to facilitate the entrance of tumor cells into the blood system[92–94]. Secondary, potentiating the EMT of CTCs[93]. Thirdly, enhancing the CTC survival in the blood system avoiding their recognition by immune cells such as leukocytes or natural killers (NK)[92,95]. Lastly, enhancing CTCs attachment to vessels, with the subsequent modifications of vascular permeability so that CTCs could extravasate and form secondary tumors[92,95]. Likewise, it has been proposed that platelets may affect drug efficacy, hence, tumor-educated platelets are being studied to develop cancer therapies[93].

2.4. Peripheral blood mononuclear cells (PBMCs) as a circulating biomarker

The breast cancer microenvironment (BCME) is composed of endothelial cells, blood vessels, immune cells, associated tissue-type cells, matrix proteins, soluble factors, etc.[96,97]. Alterations in these cells can stimulate cancer cells growth, metastasis initiation and/or response to external signals such as oxygen deprivation or therapy [96–98]. Concerning the immune system, it is described that tumor microenvironmental factors convert immune cells into tumor promoting immune cells[96]. The crosstalk between tumorigenic immune cells and cancer cells with cytokines alters the host immune system, letting tumor cells scape the immune response[83–85]. However, the interaction between the immune system and cancer cells is complex and not fully understood[98].

The function of tumor-infiltrating lymphocytes (TILs) remains controversial, but it is well-described its use as a molecular biomarker[99]. Levels of TILs, the subtype and their location correlate with prognosis and therapy response in BC [898,99]. For example, high levels of TILs in all BC subtypes predict response to neoadjuvant chemotherapy [99]. After neoadjuvant chemotherapy, TILs levels correlate with therapy response, PFS and OS in TNBC, but it is unfavorable in HR+/HER2- BC patients[99]. Concerning TILs subtype, CD4+ helper T-cells block antitumor response and promote metastasis[-98]. On the contrary, CD8+ T-cells infiltration associates with longer PFS [98,100,101].

The presence of immature dendritic cells (DC) in the BCME reduces the antitumor response for not presenting antigens to CD4+ and CD8+ T cells[98]. In addition, this immature DCs promote tumor growth due to the production of proangiogenic factors. Likewise, a higher number of infiltrated mature DCs associate with better clinical outcome and less metastasis[98,102].

Tumor-associated macrophages (TAMs) are the most abundant immune cell in tumor microenvironment. Macrophage infiltration correlated with poor prognosis and metastatic disease in human BC [103,104]. The presence of macrophages in solid breast tumors correlates with worse prognosis and chemotherapy resistance, likely activating the transcription of *STAT3*[97]. It was also observed a PI3K inhibitor resistance in *in vivo* models when the macrophage infiltration increased [97,105]. Su et *al* found that mesenchymal-like BC cells activate TAMs, what activates the EMT program in BC cell lines. Later, this crosstalk was observed in humanized

mouse models, which had worse prognosis due to the increased number of metastases[106]. Other project observed that early evolved-cancer cells recruit macrophages for cell dissemination and lung metastasis formation in HER2+ *in vivo* BC models[104]. In agreement, Qian *et al.* reported that macrophages were recruited in the pulmonary metastatic lesions, but not in the primary mammary tumor [104].

Taking into account the effect of immune cells in cancer development, several researches have tried to molecularly characterize the immune cells[96,99]. The peripheral blood mononuclear cells (PBMCs) are a novel circulating material studied as a prognostic marker in cancer[107,108]. Studies on PBMC transcriptome in cancer patients identified deregulation of immune processes such as downturned of MHC I molecules and overexpression of pro-inflammatory factors[99]. The miRNA dysregulation in PBMCs associates with tumor progression and *HER2* signaling pathways in early BC stages [107]. It is also described that the PBMCs' profile of immune-related genes predicts recurrence in TNBC[108]. Another study observed that the PBMC transcriptome had a prognostic value and correlated with clinic-pathological parameters such as tumor grade, metastasis formation and receptor status of primary tumor[109]. In a case-control study, Dumeaux V. *et al.* created a 50-gene signature considering PBMC alterations, which differentiated BC non-treated patients from cancer-free women[110]. Genetic and epigenetic alterations in PBMCs depend on cancer stage, not subtype, increasing tumor onset, progression, metastasis or drug resistance[97]. Then, tumorigenic immune cells are a prognostic factor and a potential therapeutic target[98]. Nevertheless, the results obtained studying the PBMC alterations were not robust due to the small cohort analyzed and because it was not considered the different immune cell types separately[99,107,108]. Besides, the variety of isolation techniques used reduces results'comparisson[99,107,108].

All things considered, the establishment of diagnostic markers such as tumor size, metastatic status and clinical/pathological features are not enough to guide treatment selection[20,111]. The implementation of liquid biopsy in the oncology field would facilitate an early-cancer detection as well as the development of personalized medicine[36], since it is a breakthrough technology that overcomes tumor biopsy limitations for cancer management[35]. The early dissemination of both ctDNA and CTC could be used for early detection of cancer progression and guided- patients' therapy. ctDNA mutations can identify novel targeted-markers, while CTCs can be used to test drug sensitivity, tumor aggressiveness, tumor heterogeneity, etc.[20,112–114]. Moreover, an overall understanding of tumor landscape is obtained combining the information received from different tumor-derived material, which improves patients' management. Although the molecular *in vitro* diagnostic assays increase day by day, the discordance among techniques, non-standardized protocols, the cost and the lack of sensitive techniques impaired the clinical utility of the tumor circulome[36,37]. More precise technologies as well as index or algorithms are being developed not only to understand the complex information but also to combine the data obtained from different tumor-derived material or circulating cells [35,37,115,116].

3. HR+/HER2- STAGE IV BC PATIENTS MANAGEMENT

Among all patients diagnosed with HR+/HER2- BC, 90 % have a stage I-III BC. A 20-50 % of them will have a disease progression. The remaining 5-10 % of patients are diagnosed with stage IV BC[5,9,12]. The ET has traditionally been the mainstay therapy for all stages, but patients with stage IV BC will tend to develop ET resistance earlier[117,118]. The Food and Drug (FDA) administration and the European Medicine Agencies (EMA) approved the combination of CDK4/6 inhibitors (CDK4/6i) with ET as the initial therapy for HR+/HER2- stage IV BC patients in 2017. This combination increased the PFS more than 2 years compared with ET alone [11,12,119]. Currently, there are three cyclin inhibitors approved: Palbociclib (Ibrance, Pfizer), Ribociclib (Kisqali, Novartis) and Abemaciclib (Verzenio, Eli Lilly) [120].

3.1. CDK4/6 inhibitors: mechanisms of action

The goal of treating HR+/HER2- stage IV BC patients with CDK4/6i is to inhibit the complex *CCND1-CDK4/6* to arrest the cell cycle at the G1 phase. The axis works as follow: estrogen activates the ER signaling pathway, which upregulated the expression of *CCND1*, *CDK4* and *CDK6*. The complex CCND1-CDK4/6 phosphorylates the retinoblastoma protein (pRB1), which releases E2F transcription factor. The genes activated are involved in DNA replication, leading to uncontrolled cell proliferation, as depicted in Figure 5 [9,11,12]

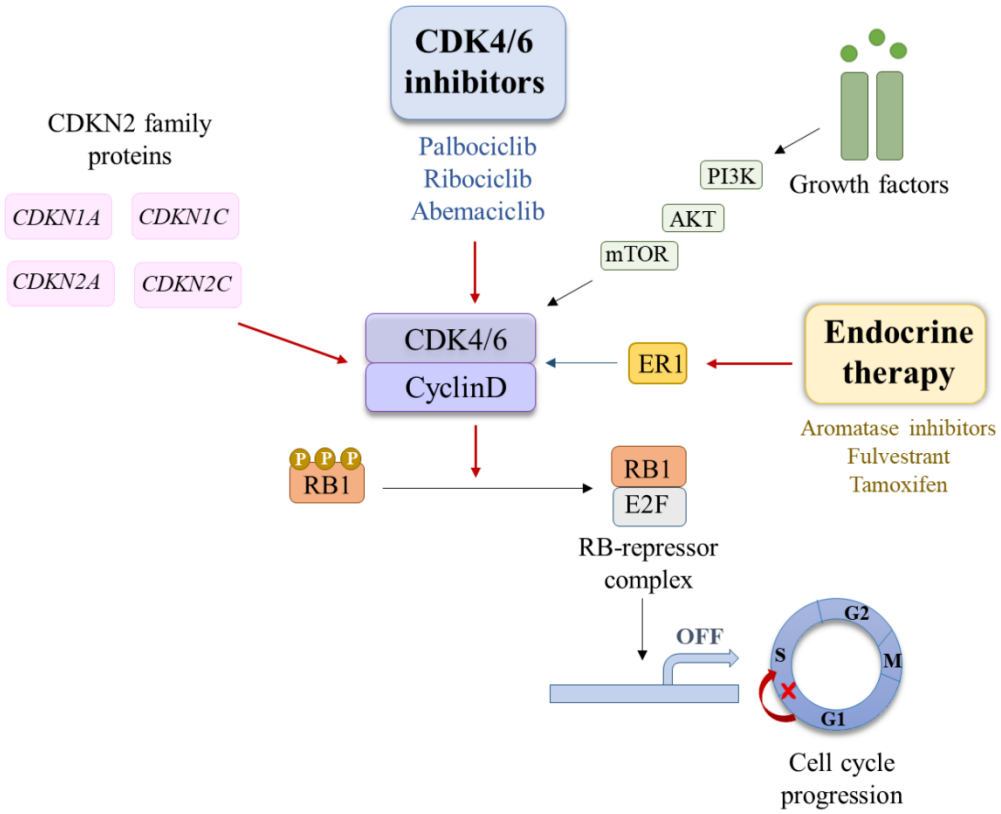


Figure 5. Cell cycle regulation in HR+/HER2- stage IV BC patients. Cell cycle is controlled by *CCND1-CDK4/6-RB1* axis. This complex is regulated by intrinsic CDK inhibitors (in pink). The current HR+/HER2- stage IV therapy is the combination of ET (in yellow) and CDK4/6 inhibitors (in blue). This polytherapy avoids the formation of the *CCND1-CDK4/6* complex. Then, *RB1* avoids the release of *E2F* transcription factor, which stops G1/S transition (*CCND1*: cyclin D1, *CDK4/6:* cyclin-dependent kinase 4/6, *RB1*: retinoblastoma, *E2F*: E2F transcription factor, CDK inhibitors: cyclin-dependent kinase inhibitors).

The first and second generation of CDK4/6i were non-specific, with limited efficacy and considerably toxicity [120]. Pharmacological and computer-aided studies improved the selectivity and inhibitory activity of these inhibitors[120]. Thus, the new generation of CDK4/6i commercially available are the previously mentioned: Palbociclib (Ibrance, Pfizer), Ribociclib (Kisqali, Novartis) and Abemaciclib (Verzenio, Eli Lilly)[119]. These drugs recognize the ATP binding domain of *CDK4* and *6*, although Abemaciclib has a superior affinity against other cyclin-dependent-kinases[120–122]. It was described that Palbociclib and Ribociclib have more than 100-fold-higher affinity for *CDK4* and *6*, while Abemaciclib has only a 6-fold higher affinity, mainly for *CDK4* than *CDK6* [12,120–122]. The differential affinity between CDK4/6i and CDKs are responsible for distinct adverse effects. The most common are neutropenia, leucopenia and diarrhea, but some rare events are being identified such as prothrombotic effect and pulmonary toxicities[119,120,123]. Concerning therapy schedule, Palbociclib and Ribociclib are administered with ET dosed daily for 21 days, needing one week break for neutrophil recovery[12,119,120]. Abemaciclib can be administered continuously every 12 hours, as monotherapy or in combination with ET [12,120,124]. In addition, Abemaciclib passes the blood-brain barrier, which might work for treating brain metastasis [12,120].

3.2. Clinical trials for the study of CDK4/6 inhibitors

To assess the clinical benefit of Palbociclib, Ribociclib and Abemaciclib in HR+/HER2- stage IV BC patients with or without previous treatments, different trials were developed (summarized in Table 1). First of all, PALOMA clinical trials (1, 2, and 3) assessed the PFS of Palbociclib in combination with ET[11,12]. The clinical benefit of this combination was also assessed in the PEARL study[125,126] and POLARIS trial[127]. Secondly, MONALEESA clinical trials (2, 3 and 7) assessed the PFS of Ribociclib in combination with ET [12,127–129]. Thirdly, MONARCH clinical trials (1 and 2) assessed the PFS and OS of Abemaciclib as monotherapy or in combination with ET[12,130–132].

Taking into account the clinical success of these inhibitors, a total of 12 CDK4/6i, including the three approved, are being tested in different clinical trials in combination with chemotherapy, immunotherapy and targeted therapies in different tumor types[133]. Likewise, several studies have shed light on the mechanisms of action of CDK4/6i, since not only induce tumor cell arrest, but also promote anti-tumor immunity [120,123].

Table 1. Clinical trials that evaluate the efficacy and safety of combining CDK4/6i plus ET in HR+/HER2- stage IV breast cancer patients (ET: endocrine therapy, AI: aromatase inhibitors, chT: chemotherapy, PFS: progression-free survival, OS: overall survival l, mo: month, NA: not applicable)

TRIAL	CDK4/6i	ET	chT	Line-therapy	PFS	OS	Menopausal status
PALOMA-1	Palbociclib	Letrozole	NA	1 st-line	26.1-5.7 mo	No-reported	Post menopausal
PALOMA-2	Palbociclib	Letrozole	NA	1 st-line	27,6-14,5 mo	No-reported	Post menopausal
PALOMA-3	Palbociclib	Fulvestrant	NA	2 nd-line	9,02-3,8 mo	34,9-28 mo	Pre and postmenopausal
PEARL	Palbociclib	Fulvestrant	Capecitabine	2 nd-line	7.5-10 mo	31.1-32.8 mo	NA
MONALEESA-2	Ribociclib	Letrozole	NA	1 st-line	25,3-16 mo	63,9-51,3 mo	Postmenopausal
MONALEESA-3	Ribociclib	Fulvestrant	NA	2nd -line	20,5-12,8 mo	53,7-41,3 mo	Postmenopausal
MONALEESA-7	Ribociclib	Tamoxifen or AI	Goreselin	2 nd- line	23.8-13 mo	No-reported	Pre-menopausal
MONARCH-1	Abemaciclib	NA	NA	2 nd- 4 th line	6 mo	17.7 mo	NA
MONARCH-2	Abemaciclib	Fulvestrant	NA	2 nd- line	16,4-9,3 mo	46,7-37,3 mo	Pre and postmenopausal
MONARCH-3	Abemaciclib	AI	NA	1 st-line	28,2-14,8 mo	No-reported	Post menopausal

3.3. Resistance mechanisms to CDK4/6 inhibitors

The vast majority of HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET develop *acquired resistance*, being the median PFS 26 months after first-line metastatic therapy. However, other patients have *intrinsic resistance* and do not respond to polytherapy [27,119]. As endocrine resistance mechanisms are not fully confirmed and since CDK4/6i acts downstream of ET (Figure 5), certain resistance mechanisms are common, complicating the study of polytherapy resistance [11,12,120]. It has been proposed some resistance biomarkers for CDK4/6i in stage IV BC patients [27,123], which can be divided as cell-cycle specific or cell cycle non-specific[27] (Table 2):

3.3.1. Cell cycle-specific resistance mechanisms

- Loss of RB1: *RB1* is a tumor suppressor gene that controls cell cycle progression[134]. In a cohort of 127 patients from the PALOMA-3 trial, no mutations were detected in *RB1* at basal. A total of 8 *RB1* mutations in the ctDNA of 6 patients were detected in Palbociclib plus Fulvestrant group at the end of treatment[70]. All variants were a stop codon or a frameshift deletion, resulting in an abnormal *RB1* function. Patients with loss of RB1 had worse PFS compare to wild-type in PALOMA-3 trial and MONALEESA 2,3 and 7 trials[27,128,129,135,136].

- CDK intrinsic inhibitors: INK family are tumor suppressor factors which avoids the formation of *CCND1-CDK4/6* complex, promoting cell cycle progression and reducing the efficacy of CDK4/6i [134]. Green *et al*. observed in cell line models that p16 (also known as *CDKN2A*) avoids the interaction between CDK4/6i and *CDK4*[134,137]. However, in the PALOMA (1, 2 and 3) trials, no differences in PFS were observed in patients with or without *CDK2A/CCND1* amplification [70,134,135,138].

- CCND1 amplification: *CCND1* controls cell cycle by regulating *CDK4*/*6* activities during G1/S phase transition. Both PALOMA 1 and 2 did not find an association between *CCND1* expression levels or amplification with Palbociclib benefit [135,138]. However, in MONALEESA-7 was observed that patients with *CCND1* alterations in the ctDNA had a shorter PFS in both therapy groups at baseline[139]. Although patients with *CCND1* altered received more benefited with Ribociclib than those patients with *CCND1* wild-type[139]. Further confirmation is needed [27].

- CDK amplification: *CDK4* and *6* play a significant role in cell cycle progression from G1 to S phase [134]. PALOMA 2 and 3 did not found evidence that *CDK4/6* amplification, mutations or changes in gene expression in the ctDNA predicted drug response. However, higher expression of *CDK6* in patients with bone metastasis associates with CDK inhibitor resistance [123]. Likewise, it was observed a *CDK6* amplification and increased *CDK6* RNA/protein levels in CDK inhibitors resistance models [70,123]. Del Re *et al.* found that patients with high *CDK4* miRNA expression in plasma-derived EVs

had longer PFS and better response to Palbociclib plus Fulvestrant, while high *CDK9* expression was associated with therapy resistance[81]

Other cell-cycle specific resistance mechanisms proposed are *E2F* amplification, increased *CDK7* expression, *cyclin-E* and *CDK2* complex formation and loss of CDK interaction protein/kinase inhibitor protein family expression [134].

3.3.2. Cell cycle non-specific mechanisms

- ER1 signaling pathway: the *ER1* mitogenic pathway is critical in the *CCND1-CDK4/6-RB1* axis [134]. In the PALOMA-3 trial, it was observed that the *ER1* mutational profile had high levels of polyclonality, since some patients lost *ER1* mutations, while others acquired them through therapy[70]. The latter associates with longer PFS in the Palbociclib group[27,70]. Focusing on single *ER1* mutations, there was a positive selection of ER1 Y537S mutation in both cohorts, which associates with longer PFS [27,70]. The data suggests that ER1 Y537s generates resistance to Fulvestrant[70]. On contrary, MONARCH-2 trial found that patients with *ER1* mutations had a longer PFS regardless of therapy group. The reasons for this discordance compare with PALOMA 3 trial are sample size, patients' criteria, analytical techniques etc. [27,131]. Lastly, PADA-1 phase III trial monitors resistance-associated *ER1* mutations in the ctDNA to tailor-patients therapy in order to improve PFS and OS before disease progression. Results are not published yet[140].

- PI3K/AKT/mTOR signaling pathway: the PI3K/AKT/mTOR axis affects estrogen signaling and consequently, the CCND1-CDK4/6-RB*1* axis[134]. *PI3KCA* mutations are common in HR+/HER2- primary BC, being clonally dominant in the majority of stage IV BC [70]. In the PALOMA 3-trial, all variants detected at basal were detected at end of treatment in both therapy groups. Likewise, the gained of *PI3KCA* truncal mutations occurs in both groups[70]. *PI3K* mutational status did not affect PFS[70,141]. Concerning the MONARCH-2 trial, patients treated with Abemaciclib had similar PFS regardless of *PI3K* mutation, although in the placebo group those patients with *PI3K* mutated had worst PFS [142]. In the MONALEESA-7 trial, it was observed a tendency in longer PFS when *PI3K* is mutated[143].

- *Fibroblast growth factor receptor* signaling: the *Fibroblast growth factor receptor (FGFR*) is involved in cell proliferation, differentiation and cell survival[134]. In PALOMA-3 trial, *FGFR2* amplification correlated with worse PFS in both therapy groups at basal[27,70]. In the MONALEESA-2 trial, patients with *FGFR2* alterations treated with Ribociclib have worse PFS, but the results were not significant probably due to the small sample size [144]. Formisano *et al.* observed that patients from MONALEESA-2 trial with higher *FGFR1* mRNA expression had shorter PFS [144]. This group also identified *FGFR1* amplification as a mechanism of ET resistance in HR+

breast tumors and in the ctDNA of HR+ patients after Palbociclib resistance [144]. In addition, they generated a CDK4/6i resistant MCF7 cell line that overexpressed *FGFR1*. The CDK4/6i resistance was revoked with the triplet combination of FGFR tyrosine kinase inhibitor Lucitanib, Palbociclib and Fulvestrant [134,144]. This triplet therapy also suppressed *CCND1*, an earlier response to estrogen and *E2F1* cell cycle transcriptional programs. The above-mentioned triplet therapy also induced a complete response in patient-derived xenografts with *FGFR*-amplified [134,144].

- *TP53* mutation: TP53-binding protein 1 (*TP53*) regulates the cell cycle inducing the transcription of antiproliferative factors such as *CDKN1A*[134]. In PALOMA-3 and MONALEESA- 2 and -7 trials, patients with *TP53* mutated had shorter PFS in both therapy groups[27,70, 139,143]. In MONARCH-3 trial, patients with *TP53* wild-type received more benefit with the polytherapy[139]. In Young Pearl phase II trial, 33 % of HR+/HER2- stage IV BC patients had *TP53* mutated in baseline tumor samples, showing worse prognosis[145].

- *KRAS* mutation: *KRAS* acts upstream of *CDK4/6*, affecting *CCND1* expression by the activation of *RAS/RAF/MEK/ERK* pathway[27,146]. In PALOMA-3 trial no association between *KRAS* mutation and PFS was detected in the ctDNA of HR+/HER2- stage IV patients[27]. On the contrary, Raimondi *et al*. found that *KRAS* mutation leads to Palbociclib intrinsic resistance and associates with shorter PFS when treated with Palbociclib plus Fulvestrant. Besides, this group also showed that the number of ctDNA copies with *KRAS* mutated correlated with the number of metastatic sites (1-, 2- or 3 metastatic locations) [27,146].

Other cell cycle non-specific resistance mechanisms are mitogen-activated protein kinase (*MAPK*) signaling pathway, overexpression of androgen receptor (*AR*), epithelial-mesenchymal transition (EMT) pathway [134] and the miR-432-5p presence in resistant to CDK4/6i patients[147].

To sum up, CDK4/6i is a new and effective therapy option to treat HR+/HER2- stage IV BC patients, but acquired resistance is inevitable. Several molecular alterations have been suggested as resistance mechanisms, but have not been clinically validated [11,123,134,148,149]. The majority of the aforementioned biomarkers are predictive of endocrine resistance, which at first does not affect sensitivity to CDK inhibitors[120, 27]. *RB1* alterations can be a prognostic and predictive biomarker of CDK inhibitor resistance, but it is not a common mutation[27,123]. *CCND1* and *KRAS* mutations might work as a prognostic and predictive biomarker of CDK4/6i, but further validation is needed [146]. Concerning *TP53*, it can be used as a prognosis biomarker, but not as predictive of polytherapy resistance[27,135,139]. *FGFR1* amplification can be a predictive biomarker of CDK4/6i resistance[144]. *ER1* and *PI3KCA* mutations detected in ctDNA are predictive of endocrine resistance, but not of CDK4/6i [70, while the use of *CDKN2A* as a biomarker is controversial. Given the clinical relevance of this therapy, a deeply research on CDK4/6i resistance mechanisms is needed to identify novel targets not only to develop new

therapy options, but also to propose new combinatorial therapies to prevent resistance or restore sensitivity[148,149].

Table 2. Summary of reported CDK4/6i resistance mechanisms in HR+/HER2- stage IV BC patients.

Genes studied	Sample	Cohort	Results	References
RB1	ctDNA at baseline and end of treatment	PALOMA-3 trial	8 mutations were detected at end of therapy in the Palbociclib group. Patients with loss of RB1 had worse PFS in the Palbociclib group.	O'Leary et al.[70]
	ctDNA at baseline	MONALEESA trial	RB1 mutation did not affect the PFS	Bertucci et al.[136]
INK family	Cell line model		CDKN2A avoids the interaction between CDK4/6i and CDK4	Green et al.[137]
	ctDNA at baseline	PALOMA trial	CDKN2A alteration did not affect the PFS	O'Leary et al.[70]
CCND1	ctDNA at baseline	PALOMA 1-2 trial	CCND1 alteration did not affect therapy benefit	O'Leary et al.[70]
	ctDNA at baseline	MONALEESA-7	CCND1 alteration associated with worse PFS Patients with CCND1 mutated received more benefit from Ribociclib	Bardia et al.[139]
CDK4	ctDNA	PALOMA 2-3	CDK4 alteration did not predict therapy response	O'Leary et al.[70]
CDK6	ctDNA	PALOMA 3	CDK6 alteration did not predict therapy response Higher CDK6 expression in patients with bone metastasis associated with CDK4/6i resistance	O'Leary et al.[70]
ER1	ctDNA at baseline and end-of-therapy	PALOMA-3	Patients with ER1 mutated at basal had worse PFS in the placebo group Patients with ER1 mutation at end of treatment had longer PFS Both therapy groups had a positive selection of Y537S, which associated with longer PFS at basal	O'Leary et al.[70]
PI3K/AKT/mTOR	ctDNA at baseline	PALOMA-3	PI3K mutation did not affect PFS	O'Leary et al.[70]
	ctDNA at baseline	MONARCH-2	Patients with PI3K mutated had worst PFS in the placebo group	Tolaney et al.

	ctDNA at baseline	MONALEESA-7	Patients with PI3K mutated had a longer PFS	Bardia et al [139]
FGFR	ctDNA at baseline	PALOMA-3	FGFR amplification associated with worse PFS	O'Leary et al. [70]
	ctDNA at progression	MONALEESA-2	Patients with FGFR mutation treated with Ribociclib had worse PFS	Formisano et al. [144]
TP53	ctDNA at baseline	PALOMA-3	Patients with TP53 mutated had worse PFS	O'Leary et al. [70]
	ctDNA at baseline	MONALEESA-2	Patients with TP53 mutated had worse PFS	Bardia et al. [139]
	ctDNA at baseline	MONARCH-3	Patients with TP53 wildtype received more benefit from the polytherapy	Bardia et al. [139]
	Baseline tumor sample	Young Pearl Phase II trial	TP53 mutated had worse PFS at basal	Lee et al. [145]
KRAS	ctDNA at baseline	PALOMA-3	KRAS alteration did not predict PFS	O'Leary et al. [70]
	ctDNA at baseline and on treatment		Patients with KRAS mutated had a shorter PFS with the combined therapy The number of mutated ctDNA copies correlated with the number of metastases	Raimondi et al.[146]

3.4.Treatment strategies after resistance to CDK4/6 inhibitor

Currently, the therapy options after resistance to CDK4/6i plus ET are not clear. The therapy-line selected will depend on the number of previous ET lines, the CDK4/6i used, the length of therapy, the metastatic size, the number of metastases, if a target mutation was found in ctDNA, etc [134,150]. The options currently manageable are summarized in Figure 6:

3.4.1. Switch the endocrine drug combined with the CDK4/6 inhibitor

It has been tested switch to another ET or CDK4/6i after progression. The nextMONARCH trial studied the combination of Abemaciclib plus Tamoxifen instead of Abemaciclib alone. No statistical differences were found in PFS, response rate and clinical benefit rate between groups [151]. Wander *et al.* proposed using Abemaciclib as monotherapy or in combination with ET after Palbociclib resistance. Both options increase PFS and OS[152]. The MAINTAIN phase II study found a longer PFS switching the ET after Ribociclib progression [134]. The EMERALD phase III study found that after polytherapy progression, Elacestrant increased the PFS time compare with Fulvestrat or AI in HR+/HER2- stage IV BC patients with 1-2 prior lines of ET and prior therapy with CDK4/6i [153].

3.4.2. Combine with targeted-therapy.

- Combined with *PI3K* inhibitor (Alpelisib). It is well-known that *PI3K* mutation confers a poor outcome in HR+/HER2- stage IV BC patients [134]. The BYLieve phase II study determined that the administration of Alpelisib plus Fulvestrant in patients whose disease progresses on CDK4/6i plus AI leads to an increased in PFS and OS when *PI3KCA* is mutated [134,154].

- Combined with *mTOR* inhibitor (Everolimus). TRINITI-1 phase I/II trial investigates the triplet combination of Ribociclib, Everolimus and Exemestane in HR+/HER2- stage IV BC patients that have progressed to CDK4/6i. The results showed the clinical benefit of the triplet therapy increasing the PFS. Additionally, it was observed that the presence of *ER1* or *PI3KCA* alternations in the ctDNA reduces the PFS [134,155].

- Combined with *STAT3* inhibitor. CDK4/6i resistance activates IL6/STAT3 pathway[155], that is involved in solid tumor progression (breast and lung cancer), metastasis formation, reduced survival, EMT, etc. [156,157]. Several approaches were proposed to target this oncogenic pathway such as targeting *JAKs* (Ruxolitinib), reducing *STAT3* expression (AZD9150 inhibitor) or targeting the phosphorylation and activation of *STAT3* (OPB compounds)[156]. The REVERT-Breast cancer phase III clinical trial is studying if the addition of a *STAT3 inhibitor* (TTI-101) reverses resistance on Palbociclib plus AI. It is estimated that the trial will be complete on February 2025[158].

3.4.3. Switch to chemotherapy:

The PALOMA-3 trial recommended chemotherapy for those patients who progressed on Palbociclib plus Fulvestrant[134]. Currently, several ongoing clinical trials are evaluating the efficacy of chemotherapy after CDK4/6i resistance (NCT04251169[159], NCT03901339[160] and NCT04134884[160]) [134]. Eribulin was evaluated as monotherapy after CDK4/6i resistance, although the data is not complete to confirm PFS and OS, it seems to provide clinical benefit [134,161]

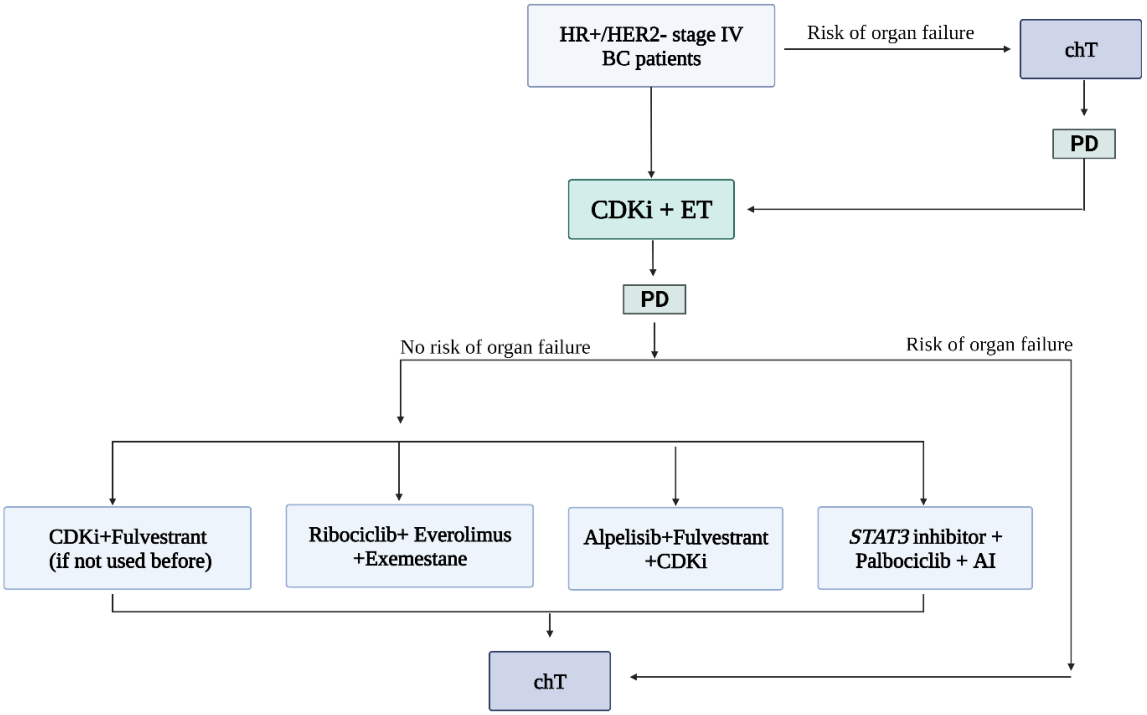


Figure 6. Treatment guideline for HR+/HER2- stage IV BC patients after CDK4/6i plus ET resistance. (HR: hormone receptor, HER2: human epidermal growth receptor, CDK4/6i: cyclin-dependent kinase 4/6 inhibitor, ET: endocrine therapy, chT: chemotherapy, PD: progression) (Source *Gennari* et al, 2021).

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On the whole, deeper research is needed to identify novel combination therapies to re-establish therapy response or develop novel therapy-lines to increase survival [134]. The decision should consider tumor characteristics at baseline, the degree of biomarker heterogeneity, prior therapies that induce the selection of resistant clones and the burden of the disease[117,118].

OBJECTIVES

OBJECTIVES

The primary aim of this thesis is to identify prognosis and predictive biomarkers in the circulating material from HR+/HER2- stage IV breast cancer patients treated with CDK4/6 inhibitors and endocrine therapy. For that, the following specific objectives have been proposed:

1. To identify biomarkers related to therapy response and resistance by analyzing the CTCs' gene expression at different time points.

2. To generate and characterize and *in vitro* model for the study of CTCs' genetic changes related to therapy resistance.

3. To identify clinically relevant mutations studying the DNA of CTC.

4. To monitor disease evolution and assess therapy response studying the cfDNA and ctDNA, as well as evaluating the clinical significance of targeted-mutations analyzing ctDNA.

5. To evaluate the clinical utility of PBMCs' transcriptome as an alternative circulating material for identifying biomarkers related to breast cancer prognosis and therapy response.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. CLINICAL SAMPLES

1.1. CTC study: characteristics of clinical samples

A total of 54 HR+/HER2- stage IV BC patients were included in this study. Patients were distributed into different cohorts as described below (see Figure S1).

1.1.1. HR+/HER2- stage IV breast cancer patient cohorts

Patient´s inclusion criteria were histologically confirmed breast carcinoma, stage IV, candidate to receive the combination of CDK4/6i plus ET as first line therapy for metastatic disease, age >18 years, disease-free interval ≥ 12 months and absence of malignant tumor current or in the last 5 years (except cutaneous carcinoma of basal cells or squamous cells or carcinoma *in situ* of the cervix adequately treated).

Considering the time of therapy response, patients were classified as *non-responders*, if disease progression occurs within 6 months after polytherapy initiation or *responders* if progression occurs after that time. The *responder* group was divided into *initial responder* if the disease progression occurs between 6 months and 2 years and *long responders* if it was after 2 years. In addition, in the study of resistance mechanisms to CDK4/6i plus ET, patients were classified with intrinsic resistance, if there is a pre-existent therapy resistance, and with acquired resistance if it is induced by a genetic alteration.

1.1.1.1. *Cohort* 1

Twenty-five patients diagnosed with HR+/HER2- stage IV BC at the Clinical Hospital of Santiago de Compostela (Spain) were recruited from February 2018 to December 2022 (Figure S1). The follow-up of patients and the clinical information were collected for 59 months. All patients gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Galicia with approval reference number 2015/772.

Regarding clinical data, the mean age at diagnose of metastatic disease was 60 years (range 40-82 years). All patients have received one previous hormone therapy before disease progression as well as CDK4/6i plus ET as first-line metastatic therapy: 16 patients have received CDK4/6i plus Letrozole, 8 patients CDK4/6i plus Fulvestrant while 1 patient has received CDK4/6i plus Anastrozole. Concerning metastatic location, 8 patients had bone metastasis, 5 had visceral metastasis and 12 had metastasis in both locations. A total of 18

patients progressed within the study in a mean time of 562 days. The *non-responder* patients (n=5) progressed in a mean time of 105 days, while the *responder* patients (n=20) in a mean time of 601 days. Those patients who initially respond to therapy (n=13) progressed in a median time of 500 days, while those with long response (n=7) at 1611 days. 10 patients died within the follow-up period, one by a different cause with no evidence of progression. The clinical data of this cohort is summarized in Table 3.

Table 3. Cohort 1: Clinical data of HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET at the Clinical Hospital of Santiago de Compostela. * One patient could not be classified as Luminal A or B by pathology analysis.

Category	n	%
Age		
< 60	11	44
> 60	14	56
Subtype		
Luminal A	10	40
Luminal B	14	56
Luminal*	1	4
Metastatic location		
Bone	8	32
Visceral	5	20
Bone & Visceral	12	48
Therapy		
Therapy (CDK4/6i)		
Abemaciclib	5	20
Palbociclib	17	68
Ribociclib	3	12
Therapy (CDK4/6i + ET)		
CDK4/6i + Letrozole	16	64
CDK4/6i + Fulvestrant	8	32
CDK4/6i + Anastrozole	1	4

For a better understanding, the cohort 1 was divided into cohort 1.a., that includes samples at disease diagnosis and after one-cycle therapy (n=21 patients at both time points), and cohort 1.b., that includes samples at disease progression (n=20 patients). It was not possible to collect a paired sample at all timepoints (Figure S1).

1.1.1.2. Cohort 2

Twuelve patients diagnosed with HR+/HER2- stage IV BC at the Clinical Hospital of Santiago de Compostela (Spain) were recruited from January 2020 to December 2022 (Figure S1). The follow-up of patients and the clinical information was collected for 36 months. All patients gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Galicia with approval reference number 2015/772.

Regarding clinical data, the mean age at diagnose of metastatic disease was 63 years (range 43-83 years). All patients have received one previous hormone therapy before disease progression as well as CDK4/6i plus ET as first-line metastatic therapy: 9 patients have received CDK4/6i plus Letrozole and 3 patients CDK4/6i plus Fulvestrant. Concerning metastatic location, 2 patients had bone metastasis, 4 had visceral metastases while 6 had metastasis in both locations. A total of 6 patients progressed within the study in a mean time of 346 days: *non-responder* patients (n=2) progressed at a mean time of 85 days while *initial* responders (n=4) at a mean time of 472 days. The other patients (n=6) did not progress and 4 patients died within the follow-up period. The clinical data of this cohort 2 is summarized in Table 4.

Table 4. Cohort 2: Clinical data of an internal validation cohort that includes HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET at Clinical Hospital of Santiago de Compostela. * One patient could not be classified as Luminal A or B by pathology analysis.

Category	n	%
Age		
< 63	6	50
> 63	6	50
Subtype		
Luminal A	5	41,6
Luminal B	6	50
Luminal*	1	8,3
Metastatic location		
Bone	2	16,6
Visceral	4	33,3
Bone & Visceral	6	50
Therapy		
Therapy (CDK4/6i)		
Abemaciclib	3	25
Palbociclib	8	66,6
Ribociclib	1	8,3
Therapy (CDK4/6i + ET)		
CDK4/6i + Letrozole	9	75
CDK4/6i + Fulvestrant	3	25
CDK4/6i + Anastrozole	0	0

1.1.1.3. Cohort 3

Sixteen patients diagnosed with HR+/HER2- stage IV BC at Essen University Hospital (German) were recruited from April 2017 to September 2020 by Kasimir-Bauer group (Figure S1). The follow-up of patients and the clinical information was collected for 42 months. All patients gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethic commission of the University Duisburg-Essen with approval reference number 12-5265-BO.

Regarding clinical data, the mean age at diagnose of metastatic disease was 56 years (range 36-86 years). All patients have received one previous hormone therapy before disease progression as well as CDK4/6i plus ET as first-line metastatic therapy: 8 patients have received CDK4/6i plus Letrozole, 7 patients CDK4/6i plus Fulvestrant while only one patient has received CDK4/6i plus Anastrozole. Concerning metastatic site, 7 patients had bone metastasis,

7 visceral metastases while 2 had metastasis in both locations. A total of 7 patients progressed within the study in a mean time of 18 months: *non-responder* patients (n=7) progressed at a mean time of 107 days, while *responder* patients (n=9) at a mean time of 635 days. Patients who initially response to therapy (n=4) progressed at 434 days, while 6 long *responders* did not progress. 6 patients died within the follow-up period. The clinical data of the stage IV BC cohort 3 is summarized in Table 5.

Table 5. Cohort 3. Clinical data of an external validation cohort that includes HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET at Essen University Hospital. *Four patients could not be classified as Luminal A or B by pathology analysis.

Category	n	%
Age		
< 56	7	43,75
> 56	9	56,25
Subtype		
Luminal A	3	18,75
Luminal B	9	56,25
Luminal*	4	25
Metastatic location		
Bone	7	43,75
Visceral	7	43,75
Bone & Visceral	2	12,50
Therapy		
Therapy (CDK4/6i)		
Abemaciclib	0	0
Palbociclib	6	37,50
Ribociclib	10	62,50
Therapy (CDK4/6i + ET)		
CDK4/6i +Letrozole	8	50
CDK4/6i + Fulvestrant	7	43,75
CDK4/6i + Anastrozole	1	6,25

1.2. PBMC study: characteristics of clinical samples

A total of ninety-eight patients were included in the study of PBMCs samples: 20 cancer-free women, 21 HR+ and 13 TNBC stage I-III patients and 26 HR+/HER2- and 18 TNBC stage IV patients (Figure S2).

1.2.1. Stage I-III breast cancer patient cohort (HR+ and TNBC): characteristics and classification

Thirty-four patients diagnosed with stage I-III BC at the Clinical Hospital of Santiago de Compostela (Spain) were included from January 2020 to October 2022 (Figure S2). All patients gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Galicia with reference number 2015/772.

Inclusion criteria were histologically confirmed breast carcinoma, stage I-III, age >18 years, no-previous neo-adjuvant therapy.

Concerning both HR+ and TNBC stage I-III patients, the mean age at diagnoses of primary tumor was 59 years (range 44-83 years). Concerning subtype, 3 patients were Luminal A, 18 Luminal B and 13 were TNBC. All patients have received chemotherapy as neoadjuvant therapy as depicted in Table 6.

Table 6. Clinical data of stage I-III HR+ (n=21) and TN (n=18) BC patients diagnosed tat Clinical Hospital of Santiago de Compostela.

Stage I-III patients		
Age	**n**	**%**
Luminal		
< 59	12	57,14
> 59	9	42,86
TNBC		
< 59	8	61,5
> 59	5	38,5
Subtype	**n**	**%**
Luminal A	3	8,82
Luminal B/HER2-	7	20,59
Luminal B/HER2+	11	32,35
TNBC	13	38,24
Therapy	**n**	**%**
Taxane	19	55,88
Taxane + Trastuzumab	8	23,52
Carboplatine + Trastuzumab	2	5,88
Others	5	14,71

1.2.2. Stage IV breast cancer patient cohort (HR+ and TNBC): characteristics and classification

A total of forty-four stage IV BC patients at the Clinical Hospital of Santiago de Compostela (Spain) were included from June 2017 to January 2023 (Figure S2). Concerning subtype, 26 patients were HR+/HER2- while 18 patients had triple negative subtype. All

patients gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Galicia with approval reference number 2015/772.

Patient´s inclusion criteria were histologically confirmed breast carcinoma, stage IV, candidate to receive CDK inhibitors or chemotherapy as first line therapy for metastatic disease according to disease subtype, age >18 years, disease-free interval ≥ 12 months and absence of malignant tumor current or in the last 5 years (except cutaneous carcinoma of basal cells or squamous cells or carcinoma *in situ* of the cervix adequately treated).

Concerning both HR+ and TNBC patients, the mean age at diagnose of metastatic disease was 61 years (range 40-85 years). Regarding metastatic location, HR+/HER2- patients had metastasis mainly both at bones and visceral (13/26) while TNBC had predominantly visceral metastasis (10/18). A total of 18 HR+/HER2- stage IV patients have received CDK4/6i plus Letrozole, 6 patients CDK4/6i plus Fulvestrant and 1 patient CDK4/6i plus AI. The TN patients have received chemotherapy: 10 patients Taxanes, 5 patients Platinum and the rest other therapy. Taking into account both subtypes, 26 out of 45 patients died during the study. Clinical data is summed in Table 7.

Table 7. Clinical data of stage IV Luminal and TNBC patients diagnosed at the Clinical Hospital of Santiago de Compostela. * Two patients could not be classified as Luminal A or B by pathology analysis.

Stage IV patients		
Age	**n**	**%**
Luminal		
< 61	15	57,69
> 61	11	42,31
TNBC		
< 61	11	61,1
> 61	7	38,9
Subtype	**n**	**%**
Luminal A	11	25,00
Luminal B	13	29,55
Luminal*	2	4,55
TNBC	18	40,91
Metastasis location	**n**	**%**
Luminal		
Bone	7	26,92
Visceral	6	23,08
Bone & Visceral	13	50,00
TNBC		
Bone	2	10,52
Visceral	10	52,63
Bone & Visceral	6	31,57
Therapy	**n**	**%**
Luminal		
CDK4/6i +Letrozole	18	69,23
CDK4/6i + Fulvestrant	6	23,08
CDK4/6i + Anastrozole	1	3,85
Only CDK4/6i	1	3,85
TNBC		
Taxane	10	55,55
Platinum	5	27,77
Other QT	3	16,66

1.2.3. White blood cell count and neutrophil-lymphocyte ratio (NLR) determination

In a blood test, the main components of white blood cells analyzed are neutrophils (40-60 % of the total white blood cell population), lymphocytes (20-40 %), monocytes (2-8 %), eosinophils (1-4 %) and basophils (0.5-1 %). In this project it has been studied the white blood cell count in 27 HR+/HER2- and 21 TN stage IV BC patients both at visit 1 and visit 2.

The ratio of neutrophil-lymphocyte (NLR) was calculated relativizing the neutrophil percentage to lymphocyte percentage both at diagnose (visit 1) and after one-cycle therapy (visit 2). A value of NLR = 1-3 implied a normal physiologic stress level, NLR =6-8 a mild physiologic stress levels, NLR =9-18 moderate physiologic stress levels and NLR>18 a severe physiologic stress level[162].

$$\text{NLR} = \frac{[\text{neutrophil percentage}]}{[\text{lymphocyte percentage}]}$$

2. BLOOD SAMPLE COLLECTION

Two EDTA-coated vacutainer tubes of 7.5 mL were collected per stage I-III HR+ BC patient before adjuvant therapy. Four EDTA-coated vacutainer tubes of 7.5 mL were collected per stage IV HR+/HER2- BC patients at different time points: when the metastatic disease is diagnosed (visit 1), after one-cycle therapy (visit 2) and at clinical progression (visit 3). Additionally, two extra EDTA-coated vacutainer tubes of 7.5 mL were collected every 3 months after visit 1 in some patients (Figure 7). For CTC counting, a CellSave preservative tube was included in some patients. Samples were processed within two hours after withdrawal.

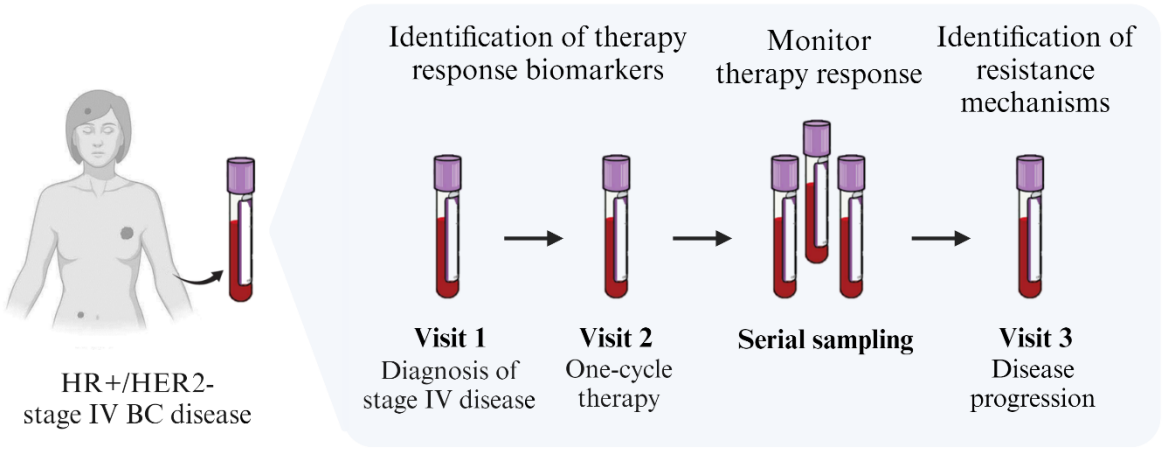


Figure 7. Scheme of blood sample collection for HR+/HER2- stage IV BC patients.

It was not possible to collect all the above-described points in all patients due to the restricted access to the hospital during the COVID-19 pandemic. Likewise, some patients quit the project and/or it was not detected a disease progression on CDK4/6i plus ET during the follow-up. A total of 21 stage IV BC patients were included before starting therapy (visit 1) and after one-cycle therapy (visit 2), while 20 patients at disease progression (visit 3). There were only 16 paired samples.

3. CTC ENUMERATION

One CellSave preservative tube (Menarini-Silicon Biosystems) was used for CTCs enumeration by CellSearch® System. This analysis was performed by trained staff from the Liquid Biopsy Analysis Unit of the Clinic Hospital of Santiago de Compostela.

The automatic process consists on incubating the fresh-peripheral blood with nanoparticles coated with antibodies anti-EpCAM. Then, CTCs are isolated and labelled with anti-CKs (-8, -18 and -19), anti-CD45 and anti-DAPI (4,6-diamino-2-phenylindole) conjugated-antibodies. Next, CellTracks Analyzer (Menarini-Silicon Biosystems) acquires digital images of the three fluorescent dyes which were reviewed to determine the CTCs count.

4. CTC NEGATIVE ENRICHMENT

CTCs were isolated from peripheral blood using a negative-enriched tetrameric antibody cocktail (RosetteSep. STEMCELL Technologies) (Table 8) with a subsequent density gradient centrifugation, what removes the blood cells and enriched the rare cells.

Table 8. List of monoclonal antibodies included in the RosetteSep™ to precipitated the unwanted cell fraction and enrich the CTCs.

Marker	Unwanted cell type
CD3	T-lymphocytes
CD14	Macrophages and neutrophils
CD16	Natural killers
CD19	Dendritic cells
CD38	Natural killers, monocytes and T- and B- lymphocytes
CD45	Leukocyte marker
CD56	Natural killers
CD61	Granulocyte
CD66b	Granulocyte
Glycophorin A	Erythroid cells

A volume of 7.5 mL of fresh blood was incubated with 500 µL of RosetteSep for 20 minutes at room temperature. Then, blood was diluted in 10 mL of phosphate buffered saline (PBS) containing 2 % of fetal bovine serum (FBS). Next, the sample was placed at a SepMate tube (STEMCELL technologies), which contained 14 mL of gradient density medium Lymphoprep (STEMCELL technologies) and a physical barrier. These tubes were centrifuged at 1200g for 20 minutes (without brake) for pelleting the unwanted cell fraction. The enriched cells were in the interface between the plasma and the density gradient medium. This interface was mixed with 10 mL of PBS-FBS 2% and transferred into another tube. Next, twice centrifuges at 1200 g for 5 minutes were performed to remove traces of the gradient density buffer (Figure S3, Supplementary material). The enriched circulating tumor cells were kept in 200 µL of cell culture media and 400 µL of RNA Later Solution (Invitrogen, ThermoFisher Scientific) and stored at -80 ºC until downstream analysis.

5. PLASMA ISOLATION

Two EDTA tubes from stage IV patients were used to isolated plasma by centrifugation.

A total of 7.5 mL of fresh blood was centrifuged at 1700 g for 15 minutes. Plasma was aliquoted and kept at -80 C until cfDNA extraction (Figure S3).

6. PBMC ISOLATION

Two EDTA tubes from cancer-free women and stage I-IV BC patients were used to isolate PBMCs by density gradient centrifugation using Lymphoprep medium in SepMate tubes.

A total of 7.5 mL of fresh blood was centrifuged at 1700 g for 15 minutes. The PBMC fraction was collected and mixed in a tube containing 10 mL of PBS-FBS 2% solution. The latter was transferred to a SepMate tube and centrifuged at 1200g for 20 minutes (without brake) to pellet the red blood cells. Next, PBMCs were recovered and centrifuged twice at 1200g for 5 minutes in PBS-FBS 2% to remove traces of the gradient density buffer. The PBMC fraction was kept in 200 µL of cell culture media and 400 µL RNA Later Solution (Invitrogen, ThermoFisher Scientific) and stored at -80 ºC until downstream analysis. PBMCs were also cryopreserved in dimethyl sulfoxide (DMSO) as backup (Figure S3).

7. NUCLEIC ACID EXTRACTION

7.1. RNA extraction

The RNA from negative-enriched CTCs and PBMCs from cancer-free women and BC patients was extracted using AllPrep DNA/RNA kit (QIAGEN), following the manufacturer's protocol. It was kept at -80 ºC until further analysis.

7.2. DNA extraction

The genomic DNA from CTCs was isolated using QIAamp DNA Micro kit (QIAGEN), following the manufacturer's protocol. The concentration was measured using Qubit 4.0 Fluorometer (Qubit dsDNA High-Sensitivity Assay Kit, Thermo Fisher Scientific). It was stored at -20 ºC until further analysis.

7.3. cfDNA isolation

After thawing 3-5 mL of stored plasma (see section 3.2.), plasma was centrifuged at 1000 g for 5 min to precipitate the debris. Then, the plasma was used to purify the cfDNA using a QIAamp Circulating Nucleic Acid kit and a vacuum system QIAvac 24 Plus (QIAGEN), following the manufacturer's protocol. Finally, it was quantified using Qubit 1x dsDNA HS assay kit (ThermoFisher). cfDNA was stored at -20 ºC until further analysis.

7.3.1. cfDNA ratio (CDR) determination

The ratio of cfDNA (CDR) was calculated comparing the cfDNA concentration at different time points to the concentration at diagnose (visit 1) [55,62]. A value of CDR>1 implied an increase in cfDNA, CDR<1 a decrease while CDR=1 no changes in cfDNA concentration.

$$CDR = \frac{[\text{cfDNA at x months}]}{[\text{cfDNA at VISIT 1}]}$$

8. NCOUNTER ANALYSIS

The NanosString nCounter is a multiplex nucleic acid hybridization technology. It was used to analyze the transcriptome of CTCs and PBMCs. For that, the PanCancer Pathway was selected since includes 770 genes involved in 13 cancer-associated canonical pathways: Wnt, Hedgehog, apoptosis, cell cycle, RAS, PI3K, STAT, MAPK, Notch, TGF-β, chromatin modification, transcriptional regulation, and DNA damage control, plus 30 PanCancer reference genes. This approach was performed at the GenVIP platform from the Foundation Health Research institute of Santiago de Compostela,

Three different assays were done. It was included the CTCs' RNA from a cohort of 6 HR+/HER2- stage IV BC patients per panel (2 patients per group: *non responder*, *initial* and *long* responder) from visit 1, 2 and 3. A pool of paired PBMC fraction was included for expression relativization.

After the nCounter analysis, a normalization analysis was performed considering positive and negative controls. Positive controls were used to calculate a normalization factor to determine the RNA counts per gene and sample. The negative controls define the minimum number of counts consider per sample.

Then, in order to determine which genes are differentially expressed among patients considering therapy response or resistance, a differential gene expression analysis was done using the DESEq2 package. A GeNorm analysis was performed to determine the best reference genes. If CTCs and PBMCs were analyzed together, *B2M* was the best reference gene. If PBMCs were analyzed alone, *TGFBR2* was the best reference gene.

9. cDNA SYNTHESIS AND PREAMPLIFICATION

In CTCs and PBMCs from stage IV BC patients, a total of 11 µL of RNA were retrotranscribed into cDNA using the SuperScript III (ThermoFisher Scientific), following manufacturer's protocol (Table 9). Due to the scarcity of CTCs in the circulation, cDNA was preamplified using Taqman Preamp Master Mix (ThermoFisher Scientific) (Table 10), containing a pool of selected TaqMan probes (Applied Biosystem) (Table S1).

In PBMCs from cancer-free women and stage I-III patients, 250 ng of RNA was both retrotranscribed and preamplified. Concerning MCF7 cell line, 1 µg of RNA was retrotranscribed.

Table 9. SuperScript III protocol (ThermoFisher Scientific) for cDNA restrotranscription

	ANNEAL	cDNA SYNTHESIS	TERMINATE REACTION	HOLD
Temperature	25 ºC	50 ºC	70 ºC	4 ºC
Time	5 min	2 h	15 min	∞

Table 10. Preamplification protocol using the selected TaqMan probes (Applied Biosystem)

	HOLD	CYCLE (14 cycles)		HOLD
Temperature	95 ºC	95 ºC	60 ºC	4 ºC
Time	10 min	15 sec	4 min	∞

10. QUANTITATIVE REAL TIME PCR

Expression was analyzed on a LightCycler 480 II (Roche Diagnostics) for a custom panel of 36 genes (TaqMan probes, Applied Biosystems) (Table S1). The RT-qPCR protocol was depicted in Table 11.

Table 11. Cycling condition for LightCycler 480 II Roche Diagnostic

		AMPLIFICATION (45 cycles)			
	INCUBATION	DENATURATION	ANNEALING	EXTENSION	COOLING
Temperature	95 ºC	95 ºC	60 ºC	72 ºC	40 ºC
Time	10 min	10 sec	30 sec	1 min	30 sec

To determine gene expression, it was calculated the median Ct value for CTCs and PBMCs. Then, the expression of CTCs and PBMCs was relativized to the reference gene previously selected using a logarithm base 2 ($2^{-\Delta Ct}$). Lastly, the difference between CTCs and paired PBMCs was subtracted to avoid PBMC background. In the cohort 3, the CTC gene expression was relativized to the reference gene and then, to *PTPRC* as a marker of non-specific isolation due to the lack of PBMC material. The expression of cell lines was relativized to the reference gene using a logarithm base 2 ($2^{-\Delta Ct}$). For MCF7 and T47D, *B2M* was selected based on previous data.

11. DNA SEQUENCING ANALYSIS

The NextSeq 500 system was used to whole-genome sequence the DNA of CTCs and PBMCs from stage IV BC patients. For that, the Human Comprehensive Cancer Panel was selected. It targets single nucleotide polymorphisms, insertions, deletions, copy number variations and gene fusions in genes commonly altered in cancer. This approach was done in the Epigenomic platform at the Foundation Health Research Institute of Santiago de Compostela. It was included the CTCs' DNA from a cohort of 6 HR+/HER2- stage IV BC patients at disease diagnosis and progression: 2 *non-responder* and *initial* responder patients at visit 1 and visit 3, while 2 *long* responder patients at visit 1. A pool of paired PBMC per patient was included to

discard germ line mutations. Then, mutations were selected considering patients group, clinical classification (benign, pathogenic or unknown) and frequency.

12. DIGITAL DROPLET PCR ANALYSIS

The quantification of somatic mutations in CTCs and ctDNA was performed by ddPCR (QX200 Droplet Digital PCR systems, Bio-RAD) in the Liquid Biopsy platform at the Foundation Health Research Institute of Santiago de Compostela. The emulsion of droplets was created using QD200 Droplet Generator (Bio-RAD). The droplets were transferred to a 96-well PCR plate. The PCR amplification was performed using C1000 Thermal Cycler (Bio-RAD) (Table 12). After amplification, the plate was read on the QX200 Droplet Reader (Bio-RAD). Finally, data analysis was performed by QuantaSoft software (Bio-RAD). Three replicated per sample were performed.

Table 12. Cycling conditions for Bio-Rad's C1000 Touch Thermal Cycler

	ENZYME ACTIVATION	DENATURATION (40 cycles)	ANNEALING	ENZYME DEACTIVATION	HOLD
Temperature	95 ºC	94 ºC	55 ºC	98 ºC	4 ºC
Time	10 min	30 sec	1 min	10 sec	∞

The *PI3K* probes were acquired at BioRAD laboratories, while the other samples were designed *ad hoc* using Primer-BLAST (NIH) and OligoAnalyzer™ Tool (IDT) (Table 13). The DNA wild-type, used as a negative control, was a genomic DNA from a cancer-free person. The positive controls were synthetic double stranded DNA fragments, called gBlock® Gene Fragments (BioRAD), which contains 10 or 50 copies of the selected mutation.

Table 13. Mutations analyzed by ddPCR in HR+/HER2- stage IV BC patients (Chr: chromosome, SNV: single number variation).

Gene	Chr	Reference Allele	Sample Allele	Variation Type	Protein Variation
SMC3	10	G	A	SNV	p.E722K
HSP90AA1	14	CCT		Deletion	p.E16del
					p.E138del
MED12	X	GCA		Deletion	p.S655del
AR	X	A	T	SNV	p.Q58L
PI3K	3	G	A	SNV	p.E542K
		G	A	SNV	p.E545K
		A	T	SNV	p.H1047L
		A	G	SNV	p.H1047R

13. IMMUNOFLUORESCENCE

PBMCs from stage I-IV BC patients were fixed with paraformaldehyde 4 % (PFA) for 15 minutes and permeabilized with InsidePerm (Miltenyi Biotec). Then, an incubation with conjugated antibodies overnight at 4ºC was done (Table 12). A flow cytometer (BD FACSAria™ III Cell Sorter) analysis was performed to identify the immune cells GZMB+/PTPRC+,that were later quantified using the FlowJo 7.6 software.

Table 12. List of conjugated antibodies used in the immunofluorescence analysis (PTPRC: protein tyrosine phosphatase receptor type C, GZMB: granzyme B).

Target	Dilution	Conjugated Ab	Reference
PTPRC	1:25	Phycoerythrin	1P-222-T100 ABCAM
GZMB	1:100	Fluor 700	AB_10561690 BD bioscience

14. CULTURE CELL LINES

MCF7 (GeneCopoeia, Inc), an estrogen-dependent human BC-derived cell line, was cultured in DMEM (Sigma Aldrich) while T47D (ATCC), a progesterone-dependent human BC-derived cell line, was cultured in RPMI 1860 (Sigma Aldrich). Both media were supplemented with 10 % FBS (Sigma Aldrich.) and 1 % penicillin-streptomycin solution (Sigma Aldrich) at 37ºC in a humidified atmosphere with 5 % CO_2.

15. PARACRINE CO-CULTURE ASSAYS

PBMCs from cancer-free women and stage I-IV BC patients were co-cultured with the MCF7 tumor cell line or culture alone as a control. Tumor cells were seeded in a 6-well cell culture plates ($2x10^5$ cells/well), whereas PBMCs ($5x10^6$ cells/well in cancer-free women and stage I-III patients; $2x10^6$ cells/well in stage IV patients) were seeded in 0.4 µm pore size tissue culture inserts. After 48 hours, both cell types were collected separately for downstream analysis.

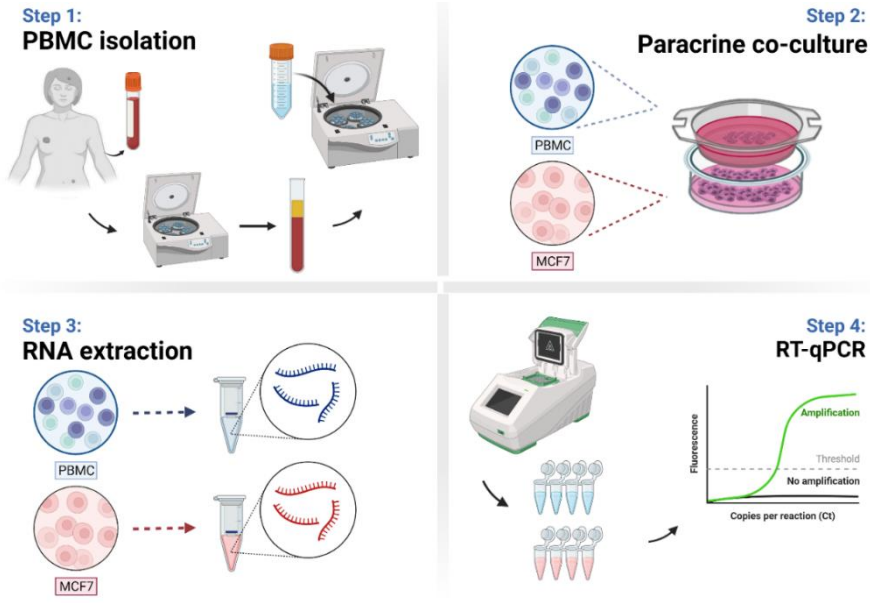


Figure 8. Workflow of paracrine co-culture assay. PBMC from cancer-free women or BC patients were isolated by density gradient centrifugation using Lymphoprep. After cell counting, 2-5x10⁶ of PBMCs were culture with 200.000 MCF7 cell lines. After 48 hours, RNA was extracted from both cell types to gene expression analysis by RT-qPCR.

16. CDK4/6i RESISTANT CELL LINE

16.1. CDK4/6 inhibitors

Two CDK4/6i (Palbociclib and Ribociclib) were obtained from Selleckchem, Palbociclib and Ribociclib. Both drugs were dissolved in DMSO and kept in – 80 ºC until use.

16.2. Determination of CDK4/6i IC50

The IC50 concentration of Palbociclib in the MCF7 cell line and Ribociclib in the T47D cell line were evaluated using the proliferative assays Thiazolyn Blue Tetrazolium Bromide (MTT) (Sigma). MCF7 cells (5000 cells per well) and T47D cells (10000 cells per well) were plated in 96-well plates. After 24 hours, escalating concentrations of Palbociclib (0.05 µM, 0.1 µM, 0.2 µM, 0.5 µM, and 0.7 µM) and Ribociclib (0.5 µM, 1 µM, 2 µM, 4 µM, and 6 µM) were added to each well respectively. In both cases, CDK4/6i were renovated every 2 days. After 15 days, drugs were removed and cell proliferation was measured using an MTT assay, following the manufacturer's instructions.

16.3. Generation of Palbociclib and Ribociclib-resistant cell line

MCF7 and T47D cell lines were treated with CDK4/6i, Palbociclib and Ribociclib respectively, for 5 months to generate resistant cell lines as depicted in Figure 9. These cells were seeded in 150 cm^2 plates (3.5-4x10^6 cells per plate). After 24 hours, when cells were over 60-70 % confluence, Palbociclib or Ribociclib was added for 2 weeks, being renovated every 2 days. Then, cells were culture without the drug for other 2 weeks. This process was repeated with the IC50 value and then, with the double of the IC50 to generate a stable population of cells growing with CDK inhibitors. Cells were collected and cryopreserved in DMSO after each dose of the CDK4/6i as a backup.

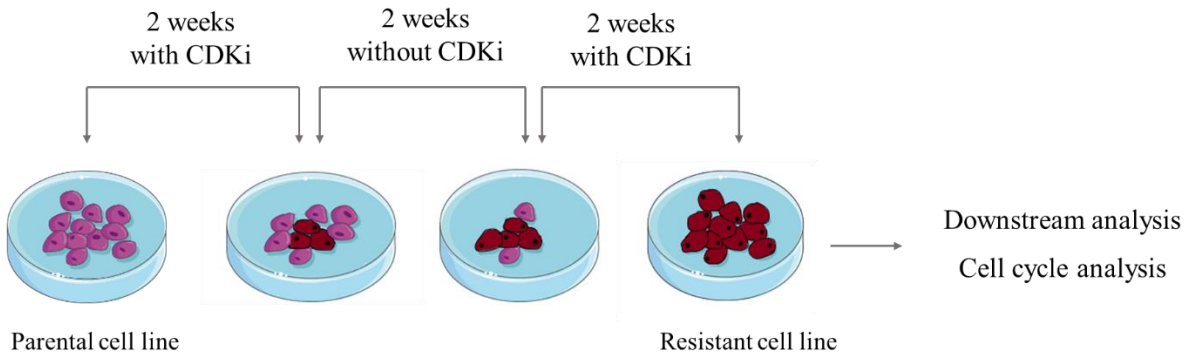


Figure 9. Workflow to generate cell lines resistant to CDK4/6i. Cell lines were treated with the IC50 of CDK4/6i for 2 weeks. Then, cells were cultured with fresh-DMEM for another 2 weeks to avoid senescence phenotype and hence, apoptosis. After the recovery time, cell lines were treated again with the IC50 of CDK4/6i for 2 weeks. This process was repeated once escalating the CDK4/6i dose to the double value of the IC50.

Parental cells were grown in parallel and exposed to DMSO as a control, mimicking the amount CDK4/6i used in the treated plates.

After each dose (IC50 and the double of IC50) tumor cells were collected and kept at -80 ºC with RNA Later for further downstream analysis. On the other hand, a million cells were fixed for cell cycle analysis, in order to confirm cell cycle retention after CDK4/6i exposure.

16.4. Proliferation assays

A total of 5.000 MCF7 Palbociclib resistant (MCF7-pR), 10.000 T47D Ribociclib resistant (T47D-rR) and their corresponding parental cell lines were plated at 96 plates. After 24 hours, the IC50 of CDK4/6i or fresh-DMEM was added. The drugs were renovated twice a week. After a week, cell proliferation was measured using MTT assay (Sigma), following manufacturer's instructions.

16.5. Colony formation assays

A total of 5.000 MCF7-pR, 10.000 T47D-rR and their corresponding parental cell lines were plated on 6-well plate. After 24 hours CDK4/6i IC50 or fresh-DMEM was added. The drug was renovated twice a week. After a week, the drug was removed. Plates were incubated for 1-2 weeks until colonies were visible under the microscope. Next, colonies were stained with crystal violet solution (Sigma-Aldrich) for manually counting.

16.6. Cell cycle analysis by flow cytometry

MCF7p-R, T47Dr-R and their parental cell lines were fixed and permeabilized with 1 mL of ethanol 70 % overnight at 4ºC. After washing with PBS, cells were stained with propidium iodide (Merck Chemicals and Life Science. S.A.). RNAse was used to avoid RNA stained. After running the cytometer (BD FACSAria^{TM} III Cell Sorter) single cell population was selected discarding clumps and doublets. Then, the percentage of cells in each cell cycle phase was quantified using FlowJo 7.6 software.

17. STATISTICAL ANALYSIS

Statistical analysis was performed using R and R Studio (version 4.3.0.) and GraphPad Prism 9.5 software (GraphPad Softwares Inc.). The Kruskal Wallis and Wilcoxon signed-rank test (for marched pairs or one-group) were used to compare the expression among patient groups or visits and parental and resistant cell lines, as well as the leukocyte levels between visits or BC subtypes. Fisher's exact test was used for association of gene expression and clinical data. PFS and OS were visualized using Kaplan-Meier plots and tested by log-rank test. A receiver operating characteristic (ROC) analysis was conducted to assess the predictive capability of the individual gene expression and cfDNA levels to classified patients according to therapy response[163], using the pROC package. Besides, a linear regression analysis was used. Then, the predict function was used to test the signature [164]. Concerning nCounter analysis, the optimal number and the most stable reference genes were selected using the geNorm package[165]. The genes differentially expressed among groups were determined using the DESEq2 package. The *long* responder group at visit 1was used as a reference group for the expression analysis. Gene Ontology enrichment analysis was performed using the STRING tool. Only p-values < 0.05 were considered statistically significant.

RESULTS

RESULTS

1. CTC STUDY: CHARACTERIZATION AND ENUMERATION

In twenty-five HR+/HER2- stage IV breast cancer (BC) patients (cohort 1, see M&M), blood samples were collected at disease diagnose (visit 1) (n=21), after one-cycle of CDK4/6i plus ET (visit 2) (n=21) and at disease progression (visit 3) (n=20). It was collected 16 paired samples at visits 1 and 3. From these samples, CTCs were negative-enriched with RosetteSep and then, the RNA was isolated to determine the CTC gene expression.

A phenotypic characterization of the CTC fraction was performed in 16 patients analyzing epithelial (*CDH1*), mesenchymal (*SNAIL1, VIM, PLS3*), stem-cell (*ALDH1A1, PROM1*) and tumor marker (*PALB2*) genes. The CTC enriched fraction showed a mixed phenotype, expressing epithelial-mesenchymal and stemness features at different time points (Figure 10). Comparing the gene expression among visits, it was observed that *ALDH1A1* has a higher expression at visit 3 compare with the other visits, although it the difference was not significant (p-value: 0.08). *PROM1* expression increased after one-cycle therapy, but it was not statistically significant. The expression of *PLS3* significantly increased at visit 3 (p-value: 0.01), while *SNAIL1* expression was significantly lower after one-cycle therapy (p-value: 0.01). *PALB2* expression was increased at disease progression, although it was not statistically significant. *CDH1* showed a stable expression among visits. Lastly, *VIM* significantly reduced the expression at visit 3 compare with visit 1 (p-value: 0.01) (Figure 10).

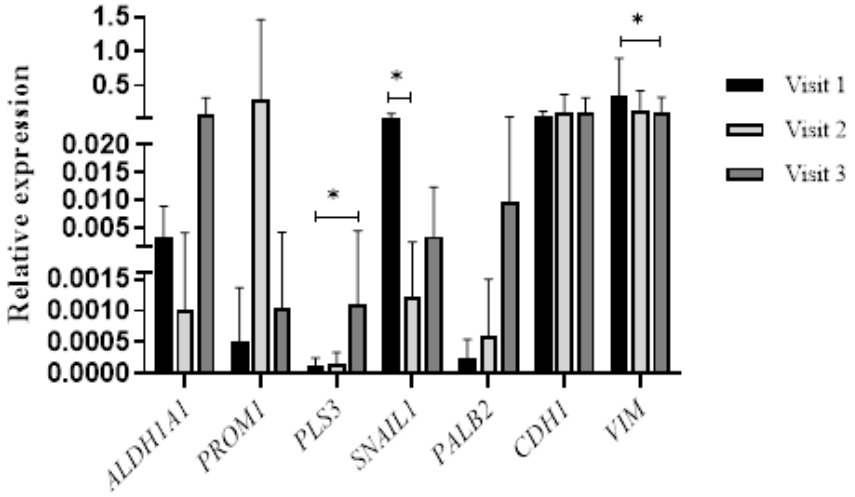


Figure 10. The CTC enriched fraction in HR+/HER2- stage IV BC patients has a mix phenotype. CTCs from 16 HR+/HER2- stage IV BC patients were isolated by Rosette Sep and gene expression was analyzed by RT-qPCR when the disease was diagnosed (visit1), after one-cycle therapy (visit 2) and at disease progression (visit 3). Statistics were performed by the paired matched samples Wilcoxon test comparing the expression among visits. (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

Next, CTCs were counted with the CellSearch® system in 12 patients at visit 1 and 2, while in 7 patients at visit 3. It was found that 9 patients (75 %) had ≥ 1 CTC and 5 patients (41, 67 %) had ≥ 5 CTCs at visit 1. After one-cycle therapy, 6 patients (50 %) had ≥ 1 CTC and 2 patients (16.6 %) had ≥ 5 CTCs. At progression, 5 patients (71.42 %) had ≥ 1 CTC and 3 patients (42.85 %) had ≥ 5 CTCs. Besides, having ≥ 1 CTC predicted worse progression-free survival (PFS) (Figure 11 A) and OS (Figure 11 B) after-one cycle therapy. The presence of ≥ 5 CTC neither predict PFS nor OS.

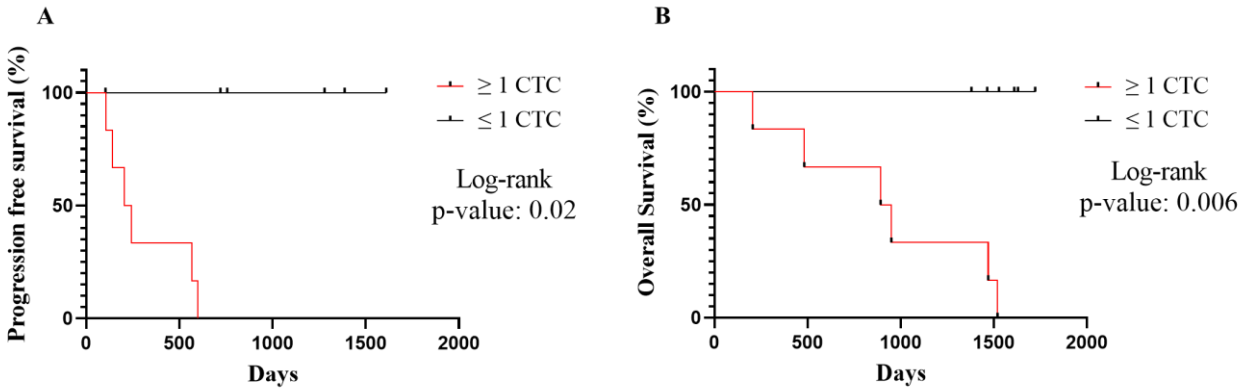


Figure 11. The presence of ≥ 1 CTC by CellSearch® associated patients' survival. Kaplan-Meier plot for A) PFS and B) OS in HR+/HER2- stage IV BC patients (n=12) after one-cycle therapy. Statistics were performed by log-rank test.

Lastly, it was checked if there was an association between having high/low gene expression (based on median value) and the number of epithelial CTCs isolated by CellSearch®. Patients with ≥ 5 CTCs had high *PROM1* expression at visit 1, but it is not significant (p-value: 0.07), while those patients with ≥ 1 CTC had a lower *ALDH1A1* expression at visit 3 (p-value: 0.04). No more associations were found.

2. CTC STUDY: PREDICTIVE BIOMARKERS

In order to study patients' response to CDK4/6i plus ET, it was analyzed the CTC gene expression before therapy (visit 1) and after one-cycle therapy (visit 2) in a cohort of 21 HR+/HER2- stage IV BC patients (cohort 1.a., see M&M).

2.1. Identification of therapy response biomarkers in CTCs

A gene discovery study was performed using the Nanostring nCounter technology. It was analyzed the CTCs' RNA from 6 patients (2 non-responder, 2 initial-responder and 2 long-responder patients) at both visit 1 and 2. It was chosen the PanCancer Pathway Panel since includes 770 genes from 13 cancer-associated canonical pathways. Likewise, a pool of

autologous PBMCs was included as a control for each patient. Then, a differential gene expression (DGE) analysis was done using the DESEq2 package to determine which genes were differentially expressed among the groups analyzed. The *long responder* group was used as the control group.

After the DGE analysis, it was found that 31 genes were differentially expressed at disease diagnosis (Figure 12 A) and 153 genes after one-cycle therapy (Figure 12 B).

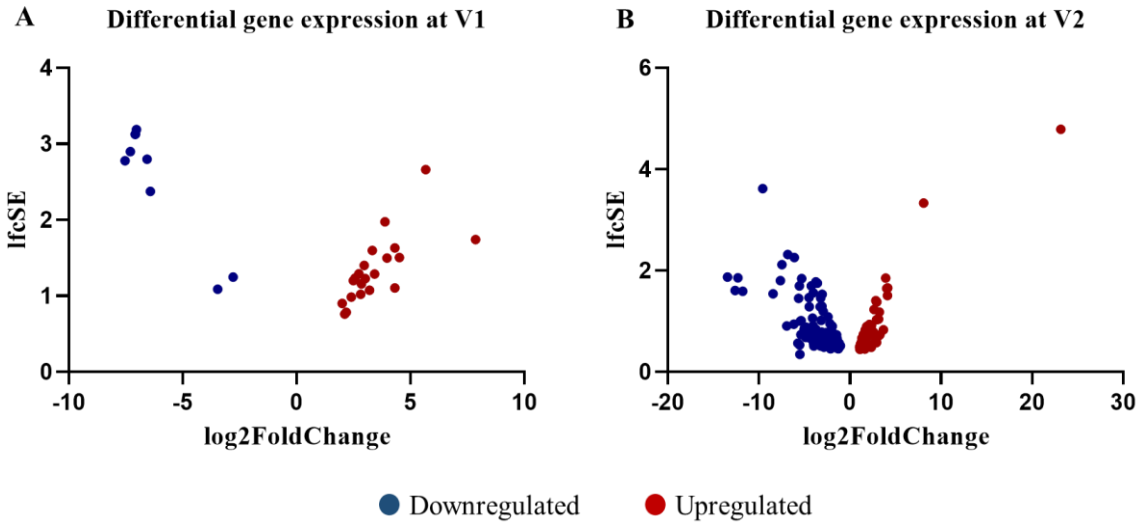


Figure 12. Volcano plot of CTCs' RNA counts from the nCounter analysis after DGE analysis, using the *long responder* patients as a reference group. A) DGE when the metastatic disease was diagnosed (visit 1), B) DGE after one-cycle therapy (visit 2). Downregulated genes are depicted in blue while upregulated genes in red. Statistics were performed by the DESEq2 package.

Next, a Gene Ontology (GO) enrichment analysis was performed with the aforementioned genes. At diagnosis of the metastatic disease, the genes differentially expressed are mainly involved in regulation of immune response and apoptotic pathways, while after one-cycle therapy, genes differentially expressed are involved in cell communication and signal transduction pathways (Figure S4).

After the gene discovery study and the differential expression analysis from 6 patients, a total of 16 genes were selected for validation. Genes related with CCND1-CDK4/6 axis were also included (Table S1). Gene expression validation was done by RT-qPCR in 21 HR+/HER2- stage IV BC patients. CTC gene expression was relativized to *B2M* expression and normalized with paired PBMC expression.

First, the expression of CTCs was compared between *non- responder (n=5)* and *responder* (n=16) patients before starting therapy (visit 1). *Non-responder* patients showed a higher expression of *PRKCB* (p-value: 0.011), *MAPK3* (p-value: 0.006) and *STAT3* (p-value: 0.008) (Figure 13 A-C), while *responder* patients had a higher expression of *CDK6* (p-value: 0.011) and *CCND1* (p-value: 0.035) (Figure 13 D-E). Although the expression of *CDK4* was not significantly different between groups, further examination of the *responder* group showed that CTCs from *long* responders had a higher *CDK4* expression than *non-responder* patients (p-

value: 0.02) (Figure 13 F). No differences were observed in *CDK4* expression between *initial* responder and *long*- or *non-responder* patients (p-value > 0.05).

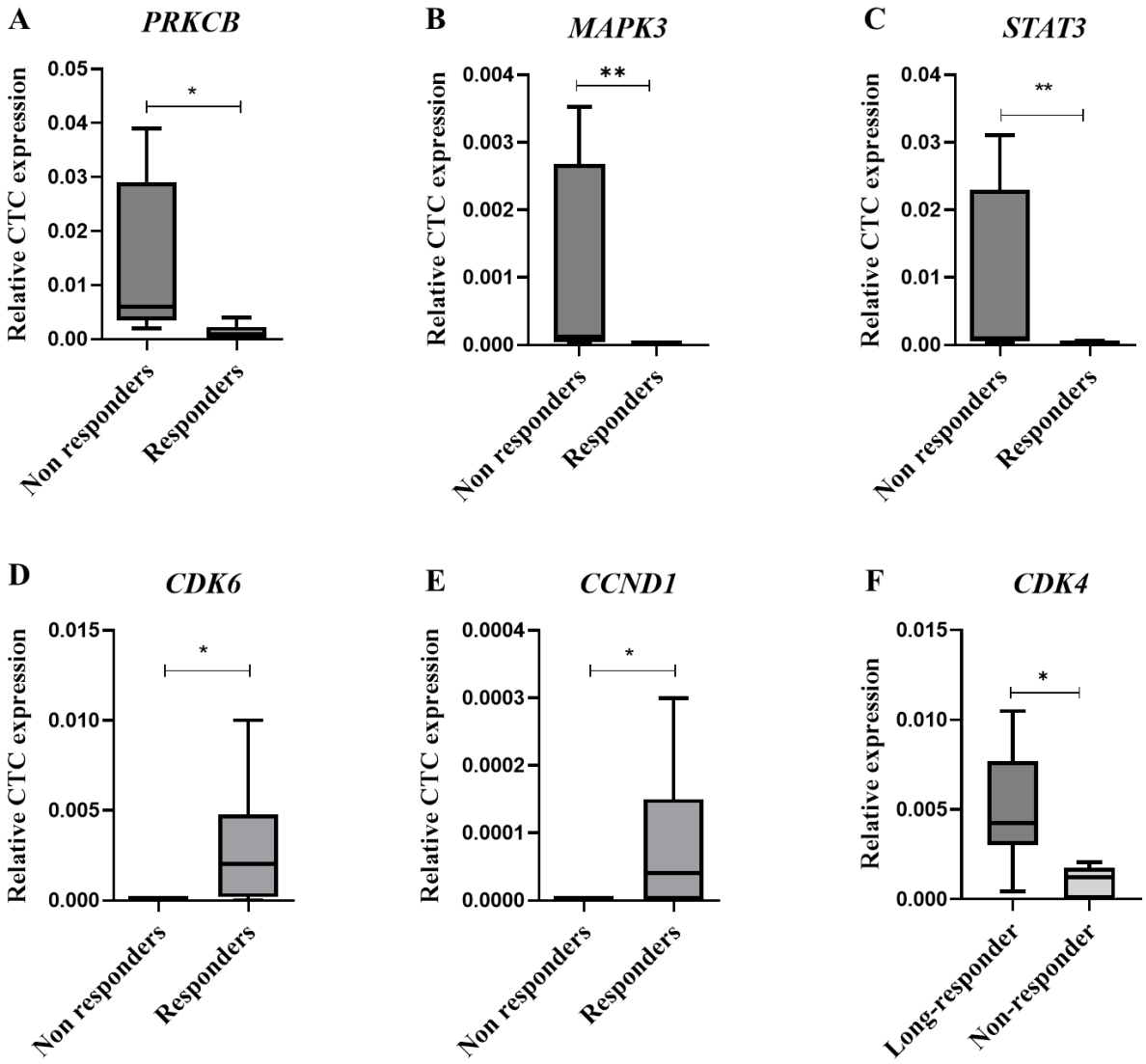


Figure 13. CTC gene expression from *non-responder* (n=5) and *responder* (n=16) patients at visit1 1.A) *PRKCB*, B) *MAPK3*, C) *STAT3*, D) *CDK6*, E) *CCND1* and F) *CDK4*. CTC gene expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

Then, if the *responder* group was further examined, it was detected differences in the CTCs' gene expression between *initial* and *long responder* patients. Thus, the *initial-responder* group had a higher expression of *CCND1* (p-value: 0.04) (Figure 14 A) while the *long responder* group had a higher expression of *DUSP5* (p-value: 0.01) (Figure 14 B).

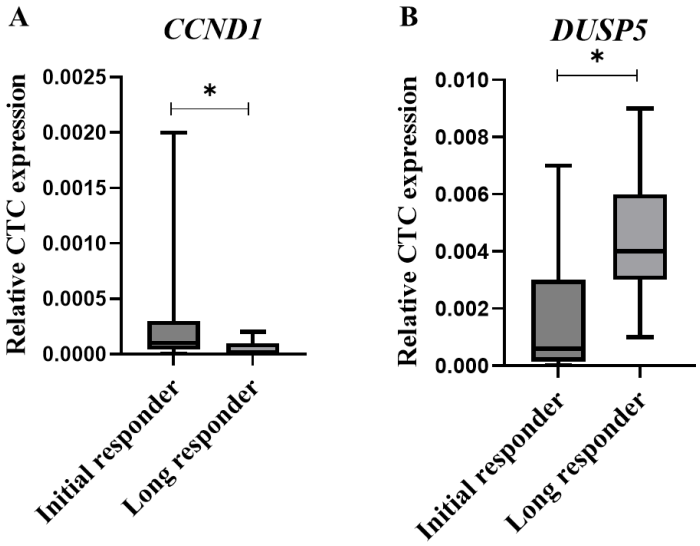


Figure 14. CTC gene expression from *initial* (n=9) and *long* responder (n=7) patients at visit 1. A) *CCND1* and B) *DUSP5*. CTC gene expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

Lastly, it was evaluated the expression of genes differentially expressed between *non-responder* and *responder* patients after the first-cycle therapy (visit 2). CTCs from *responder* patients had high expression of *CDH1* (p-value: 0.01) (Figure 15 A), while *CUL1* (p-value: 0.03) and *CDKN1C* (p-value: 0.03) had a higher expression in *non-responder* patients (Figure 15 B-C). No differences were found when the *responder* group was divided into *long* and *initial* responders.

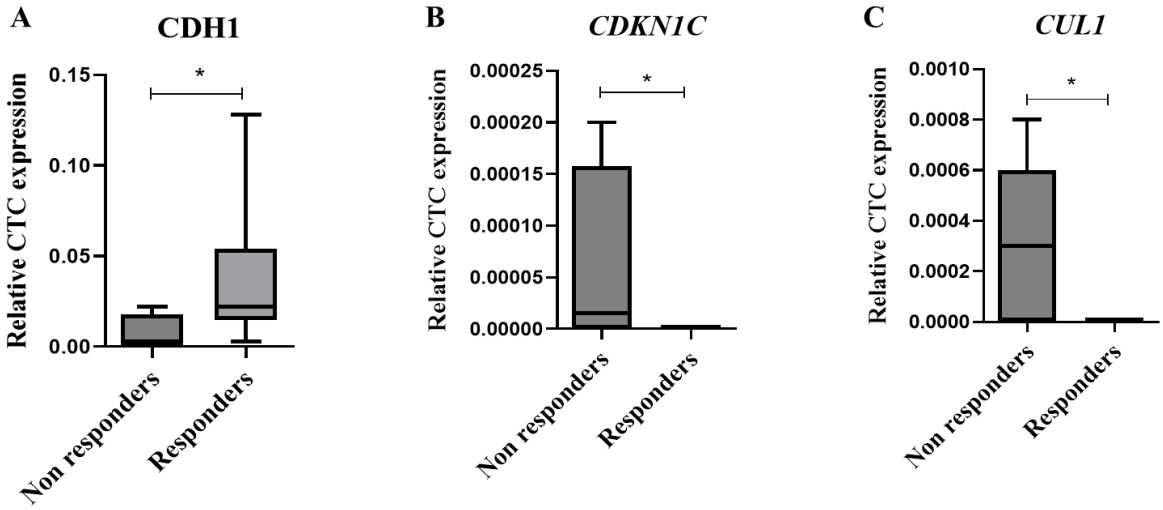


Figure 15. CTC gene expression from *non-responder* (n=5) and *responder* (n=16) patients at visit 2. A) *CDH1*, B) *CDKN1C* and C) *CUL1*. CTC gene expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

After one-cycle therapy, high levels of *CDK4* were associated with low levels of *CDKN1C* (p-value: 0.03). Likewise, there is a positive association between *CCND1* and *CDKN2C* expression (p-value: 0.04). Moreover, there was a positive association between *STAT3* and *MAPK3* (p-value: 0.003). There was a tendency towards a positive association between *STAT3* and *PRKCB* (p-value: 0.08) as well as *PI3KCG* and *PRKCB* (p-value: 0.08). Based on the contingency analysis data, a linear regression analysis was performed to determine the correlation between these genes. It was only found a strong correlation between *STAT3* with *PRKCB* (p-value: 5.7×10^{-6}, R^2: 0.97).

A contingency analysis was done to investigate if the gene expression of CTCs associated with the clinical characteristics of our cohort (n=21) before starting polytherapy (visit 1). The median gene expression values were used as a cutoff to define high/low expression levels. Results showed that patients with lung metastasis displayed high *PLS3* levels (p-value: 0.03). No other significant association was found.

In order to determine the association among the genes analyzed from CTCs, a contingency analysis was conducted. The median values were used as a cutoff to define high/low expression levels. Before starting therapy, it was observed that high *SNAIL1* expression was associated with high *VIM* expression (p-value: 0.008). Although it was not significant, there was a tendency between having high levels of *SNAIL1* and high levels of *PI3KCG* and *PI3KCA* (p-value: 0.08). It was also found a positive association between *PI3KCG* with *JAK2* (p-value: 0.0003) and *CUL1* (p-value: 0.004), as well as between *STAT3* and *PRKCB* (p-value: 0.008). Although it is not significant (p-value: 0.08), there is a tendency towards a positive association between *STAT3* and *MAPK3* (p-value: 0.08) and *PRKCB* and *MAPK3* (p-value: 0.08). Concerning stem cell markers, there was a positive association between *ALDH1A1* and *PLS3* expression (p-value: 0.02). Regarding the *CCND1-CDK4/6* axis, there is a positive association between *CDK4* and *CDK6* (p-value: 0.008). There is a tendency towards having high levels of *PI3KCG* and *RB1* (p-value: 0.08) as well as *CDK6* and *PI3KCG* (p-value: 0.08).

2.2. A CTC gene expression signature to classified CDK4/6i *non-responder* patients

To test if the CTC gene expression classified patients according to therapy response, a Receiver Operating Characteristics (ROC) analysis was performed with genes differentially expressed between groups at visit 1. *STAT3*, *PRKCB* and *MAPK3* for the *non-responder* and *CDK6*, *CDH1*, *CCND1* and *DUSP5* for the *responder* group. The expression of each gene analyzed independently did not classified patients considering polytherapy response (AUC < 0.7). After testing different gene combinations, the signature that includes $STAT3^{high}$, $PRKCB^{high}$ and $CDK6^{low}$classified all the *non-responder* patients with an Area Under the Curve (AUC) of 1 (Figure 16 A).

This gene expression signature was next validated in two cohorts of HR+/HER2- stage IV BC patients treated with polytherapy as first-line metastatic treatment. The first cohort, denominated as cohort 2, was an internal validation cohort that included 12 stage IV BC patients (2 *non-responders* and 10 *responder* patients). The CTC gene expression was analyzed as

previously described (section 3, see M&M). Then, a predictive function was used to confirm whether the gene signature classified patients according to therapy response. The results were promising since a total of 11 out of 12 patients were correctly classified, achieving a model success rate of 92 %. The second cohort, denominated as cohort 3, was an external validation cohort that includes 16 stage IV BC patients (7 *non-responder* and 9 *responde*r patients) from Essen University Hospital. In this case, the CTC fraction was isolated using the Adnatest, based on the expression of *EpCAM*, *EGFR* and *HER2*. Then, CTC expression was relativized to *PTPRC*, instead of subtracting PBMC expression due to the lack of this material. Based on the *STAT3*, *PRKCB* and *CDK6* CTC expression, the ROC analysis yields an AUC=0.83 (Figure 16 B).

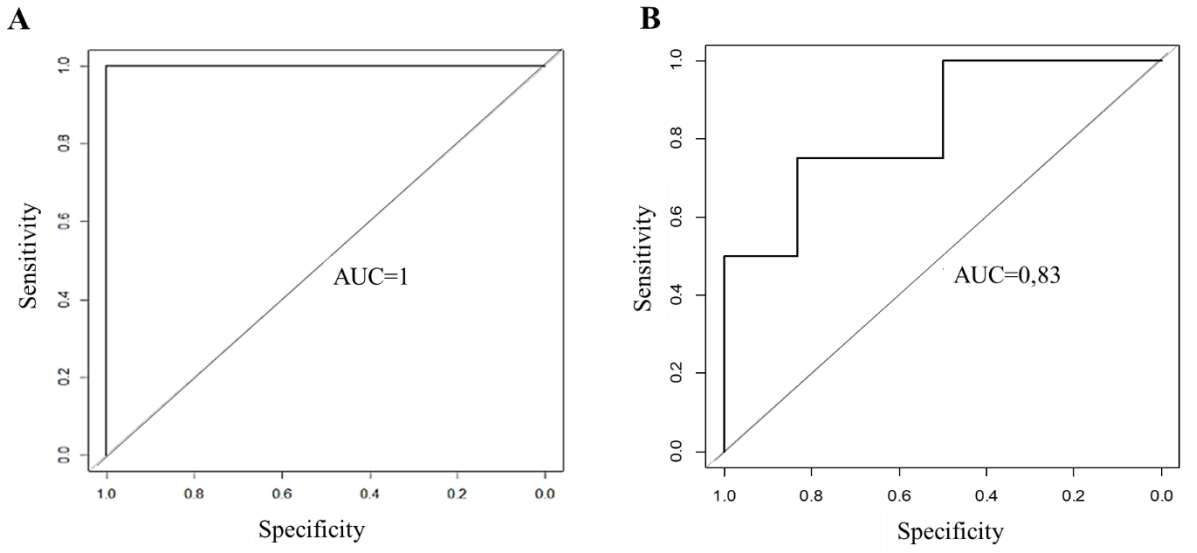


Figure 16. The CTC genetic signature obtained by analyzing the expression of *STAT3*, *PRKCB* and *CDK6* classified CDK4/6i non responder patients accurately. A) The CTC genetic signature classified all patients from *cohort 1.a*. according to therapy response at visit 1 with an AUC=1 (n=21, 5 *non-responder* vs 16 r*esponder*). B) The CTC genetic signature classified patients from *cohort 3* according to therapy response at visit 1 with an AUC=0.83 (n=16, 7 *non-responder* vs 9 *responder*).

2.3. Identification of prognostic biomarkers at therapy initiation and after one-cycle therapy in CTCs

The expression from the previous analyzed genes was consider to performed a Kaplan-Meier estimator (long rank test) to evaluate the PFS and OS in the HR+/HER2- stage IV BC patients treated with the polytherapy (cohort 1.a., n=21 patients). The median values of CTC gene expression were used as a cutoff to define high/low expression levels. A total of 18 out of 21 patients had progressed while 8 out of 21 had died during the follow-up.

Before starting therapy (visit 1), higher expression of *DUSP* (757 days vs 342 days, p-value: 0.01 by log-rank test) (Figure 17 A) and *CDK4* (829.5 days vs 243 days, p-value: 0.0005 by log-rank test) (Figure 17 B) associated with longer PFS. Moreover, combining the expression of *CDK4* and *STAT3 (CDK4^{low}STAT3^{high})*, the potential of discriminate patients with worse PFS was enhanced (141 days vs 757 days, p-value: < 0.0001 by long rank test) (Figure

17 C). Regarding OS, higher levels of *PALB2* associated with longer OS (undefined vs 899 days, p-value: 0.008 by log-rank test) (Figure 17 E). After one-cycle of therapy (visit 2), low *CDKN1C* expression (166 days vs 721 days, p-value: 0.02 by log-rank test) (Figure 17 D) associated with longer PFS.

Additionally, a survival analysis was conducted to assess the impact of various therapies (CDK4/6i plus ET), patient's age, metastatic locations and the number of metastases. However, no significant associations were identified for none of these parameters. Thus, clinical data was not taken into account for the subsequent Cox regression analysis.

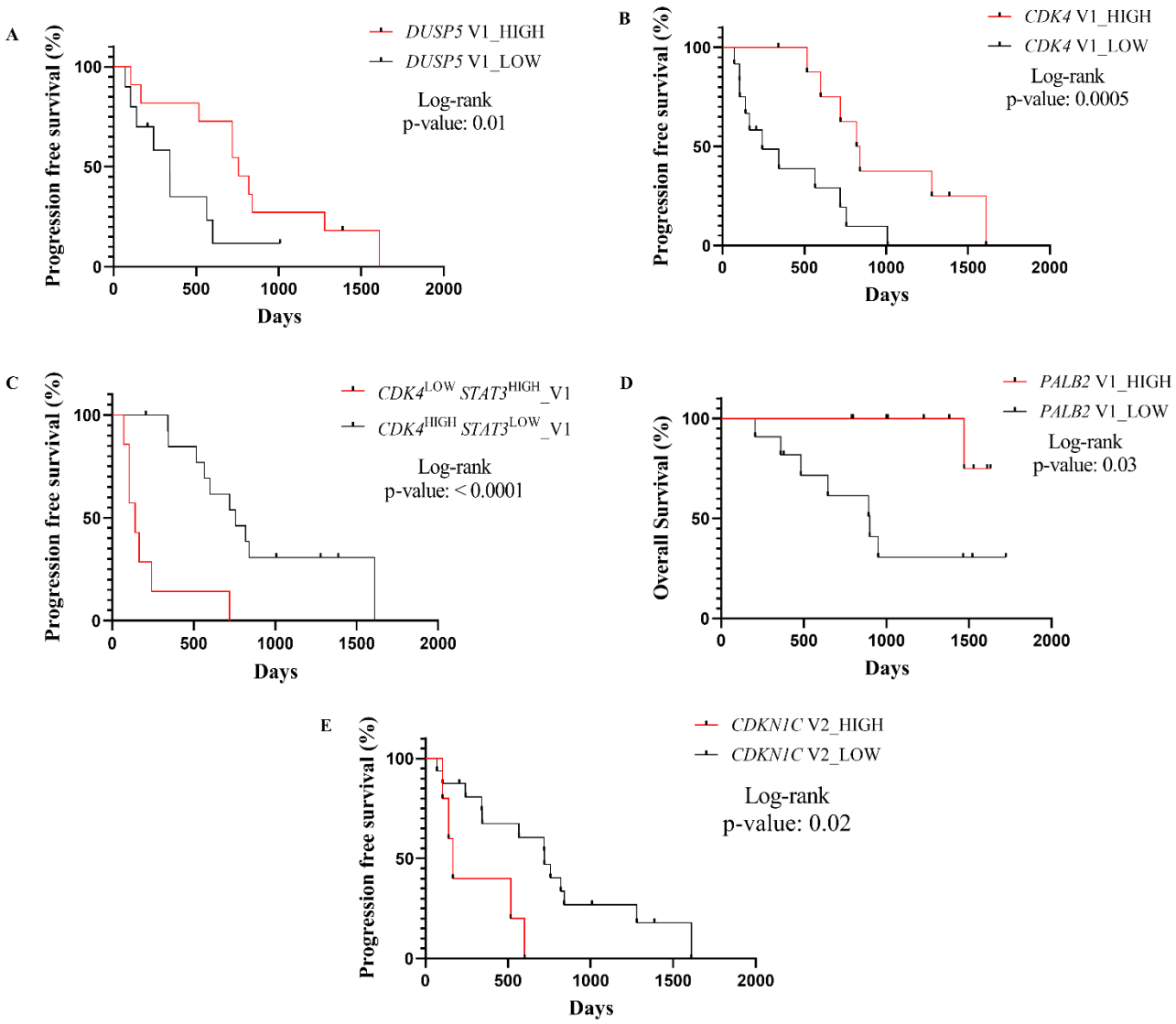


Figure 16. CTC gene expression associated with patients' survival in the cohort 1.a. Kaplan-Meier plot for PFS of A) *DUSP5* expression at VISIT 1, B) *CDK4* expression at visit 1 and C) CDK4 and *STAT3* expression at visit 1. Kaplan-Meier plot for OS of D) *PALB2* expression at visit 1 and E) *CDKN1C* expression at visit 2. Statistic was performed by Log-rank test. High or low values were calculated considering the median expression.

2.4. Immune cells non-depleted in the CTC-enriched fraction

It was also observed that *Granzyme B* (*GZMB*) expression was significantly higher in *long* responder patients compared with those patients whose disease progressed (*initial* responders and *non-responders*) (p-value: 0.03) (Figure 18 A). Likewise, high *GZMB* expression in the CTC-enriched fraction associated with longer PFS (788 days vs 342 days, p-value: 0.023 by log-rank test) (Figure 18 B). The *GZMB* protein is secreted by natural killers (NKs) and lymphocytes T cytotoxic [166]. As some immune cells are non-specifically isolated along with the CTC fraction (Section S1, Figures S5 and S6), it is likely that the above-mentioned results could be attributed to the expression of PBMCs rather than CTCs.

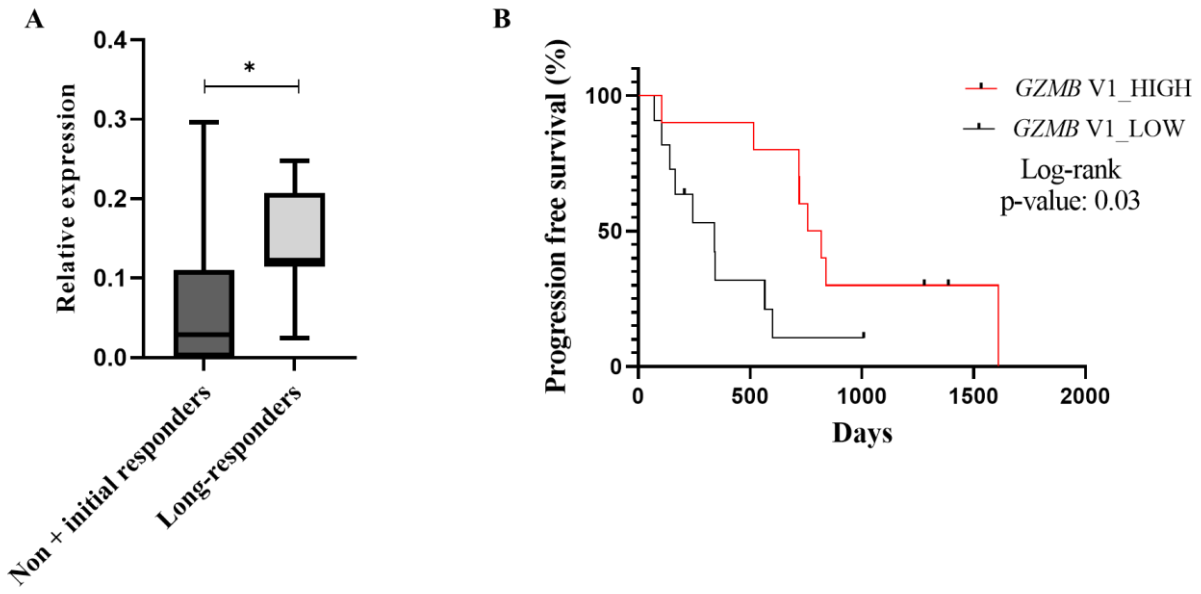


Figure 18. *GZMB* expression in the CTC-enriched fraction from the cohort 1.a. associated with A) therapy response and B) PFS at VISIT 1. CTC expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. High/low values were calculated considering the CTC median expression. Statistics were performed by Wilcoxon test and log-rank test. (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

2.5. Identification of resistance biomarkers in CTCs

In order to identify resistance mechanisms to CDK4/6i plus ET, the CTC gene expression was analyzed when the metastatic disease was diagnosed (visit 1) and at radiological progression (visit 3) in a cohort of 20 HR+/HER2- stage IV BC patients (cohort 1.b., see M&M). Paired blood samples (visit 1- visit 3) were obtained in 16 patients, while in 4 patients the blood sample was collected only at disease progression.

As in section 2.1., a gene expression discovery study was performed using the Nanostring nCounter technology (PanCancer Pathway Panel). It was analyzed the CTCs' RNA from 6 patients (2 patients with intrinsic resistance and 4 patients with *acquired resistance*) at visit 3. Likewise, a pool of autologous PBMCs was included as a control for each patient. Then, a DGE analysis was done to determine which genes were differentially expressed at visit 3 comparing

with visit 1. For that, the data from *long responder* patients at visit 1 was used as the control group. A total of 33 genes were differentially expressed at disease progression (Figure 19).

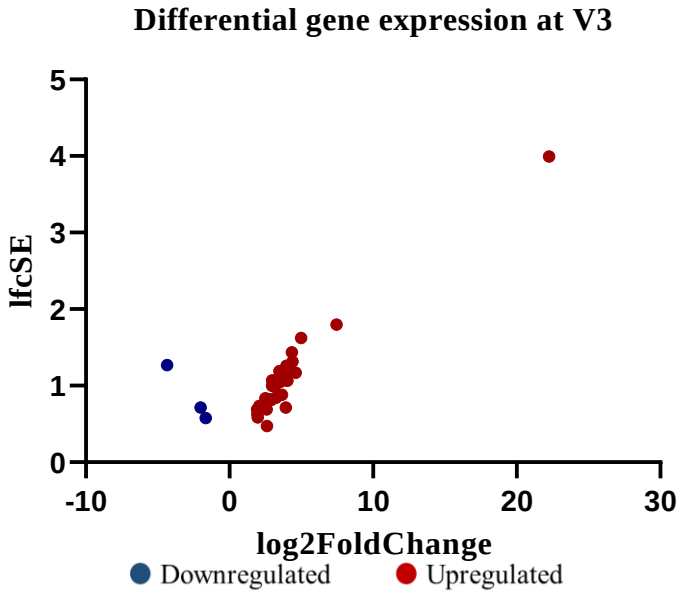


Figure 17. Volcano plot of CTCs' RNA counts after differential gene expression analysis at disease progression (visit 3), using the data from *long* responder patients at visit 1 as a reference group. Downregulated genes are depicted in blue while upregulated genes in red. Statistics were performed by the DESEq2 package.

Next, a GO enrichment analysis was performed with the 33 genes previously selected. At disease progression, the genes differentially expressed were involved in regulation of gene expression. Likewise, the number of genes involved in the regulation of cell proliferation, immune response and development enhanced comparing whit the visit 2. In addition, the number of genes related to regulation of gene expression and epigenetic factors was higher at visit 3 compare with the other visits (Figure S4).

After the DGE analysis, 8 genes were selected for validation by RT-qPCR (Table S1). Besides, genes related to CCND1-CDK4/6 axis and genes differentially expressed between groups at visit 1 and visit 2 were included (Table S1). As previously said, CTC gene expression was relativized to *B2M* and normalized with paired PBMC.

In order to determine the acquired resistance mechanisms to polytherapy, the CTC expression was compared before starting therapy (visit 1) and at disease progression (visit 3) in *responder* patients (n= 16 patients at visit 1 and visit 3). Non-responder patients were excluded from the analysis due their presumed intrinsic resistance. It was observed a higher expression at progression of *BAX* (p-value: 0.02), *EZH2* (p-value: 0.00005), *HDAC6* (p-value: 0.00003), *PLAU* (p-value: 0.01), *RELA* (p-value: 0.005) and *CDC7* (p-value: 0.00005) (Figure 20 A-F), while a lower expression of *NFKBIα* (p-value: 0.002) and *SNAIL1* (p-value: 0.01) (Figure 20 G-H).

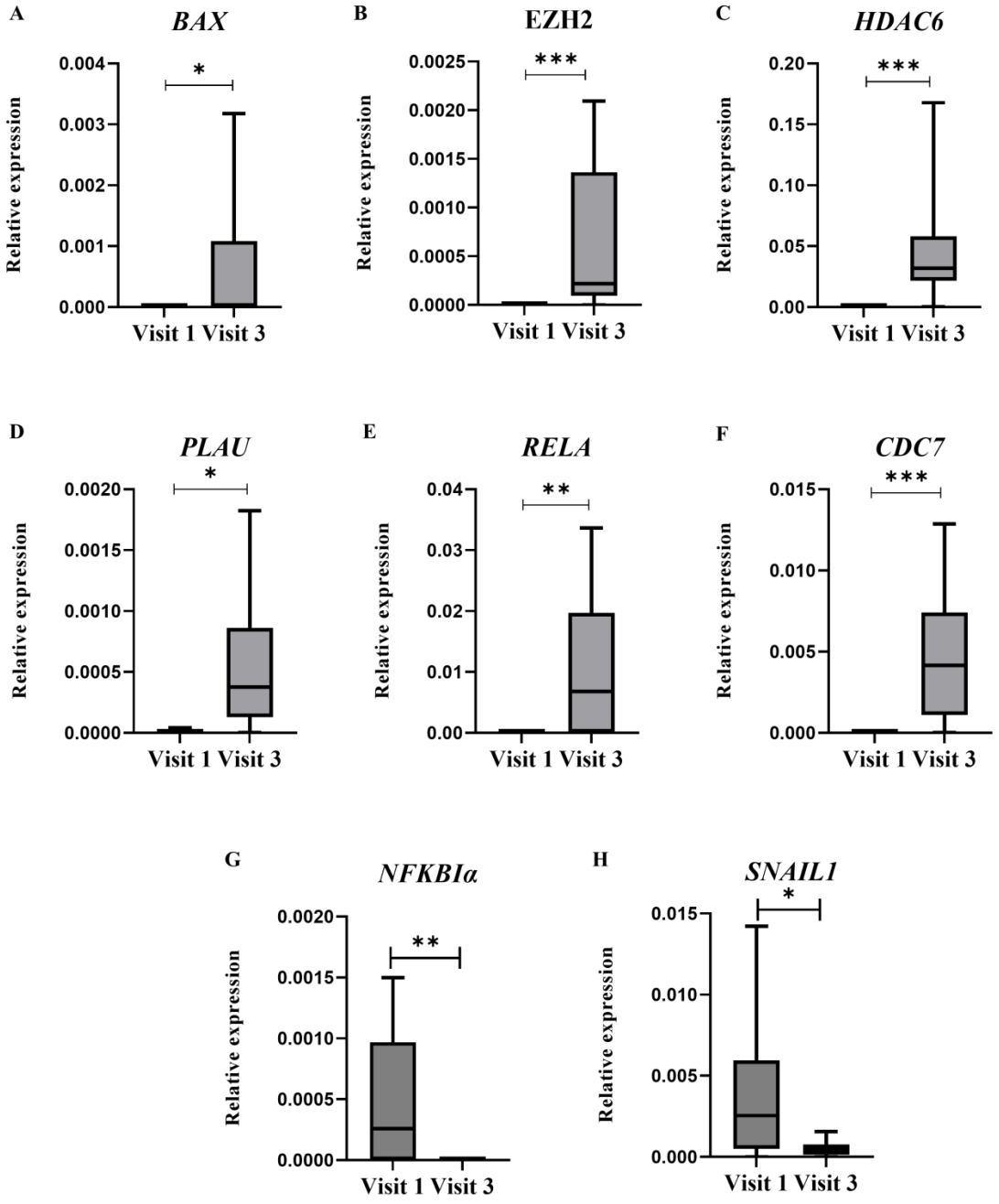


Figure 18. CTC gene expression differs between visit 1 and visit 3 in patients with acquired resistance (n= 16). A) *BAX*, B) *EZH2*, C) *HDAC6*, D) *PLAU*, E) *RELA*, F) *CDC7*, G) *NFKBIa* and H) *SNAIL1*. The expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by paired-matched Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

Moreover, if the *responder* group is divided, there were differences in gene expression between *initial* (n=12) and *long* responder (n=4) patients at disease progression. Thus, the *long* responder patients showed a higher expression of *CDK6* (p-value: 0.03) (Figure 21 A) and *PROM1* (p-value: 0.03) (Figure 21 B). The difference in *BAX* and *CDC7* expression was almost significant (p-value: 0.08 and 0.07, respectively).

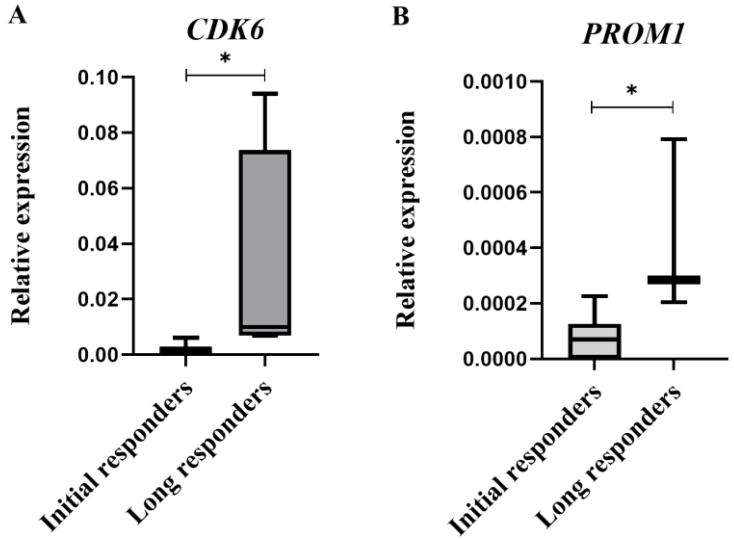


Figure 19. CTC gene expression from *initial* responder (n=12) and *long* responder (n=4) patients at disease progression. A) *CDK6* and B) *PROM1*. The expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

Next, in order to confirm if the expression of the aforementioned genes was altered due to resistance acquisition, it was determined their expression in *non-responder* patients, it means, in patients with intrinsic resistance. Of the analyzed genes, it was found differential expression levels in three of them. Thus, a higher expression of *HDAC6* (p-value: 0.04) and *CDC7* (p-value: 0.03) (Figure 22 A-B) was found in patients with *acquired* resistance, while high *CASP8* expression in patients with *intrinsic* resistance (p-value: 0.03) (Figure 22 C). The difference in *CCND1* is near significance, being higher in patients with acquired resistance (p-value: 0.06).

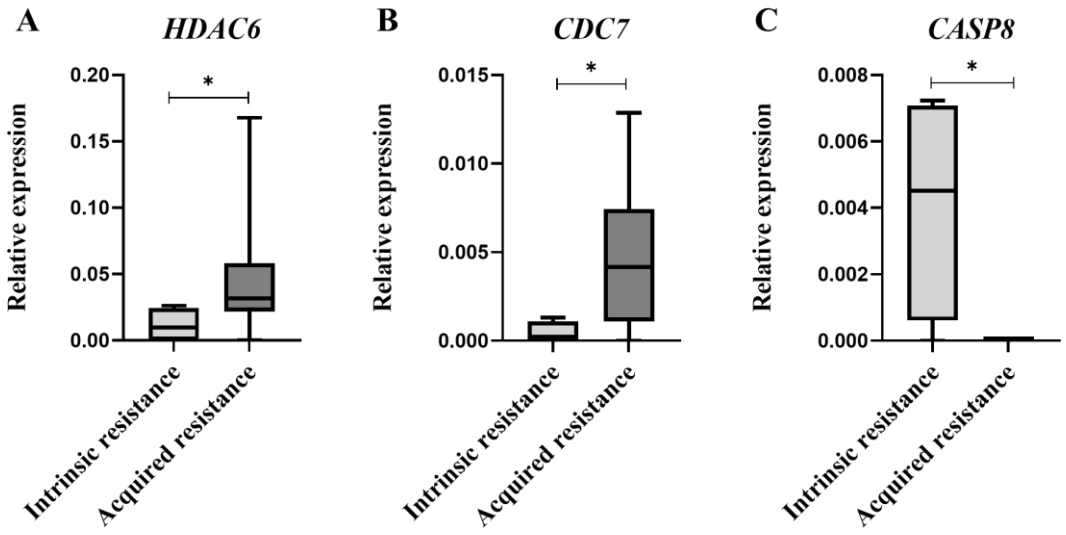


Figure 20. CTC gene expression from patients with intrinsic (n=5) or acquired resistance (n=16) at progression. A) *HDAC6*, B) *CDC7* and C) *CASP8*. The expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***)).

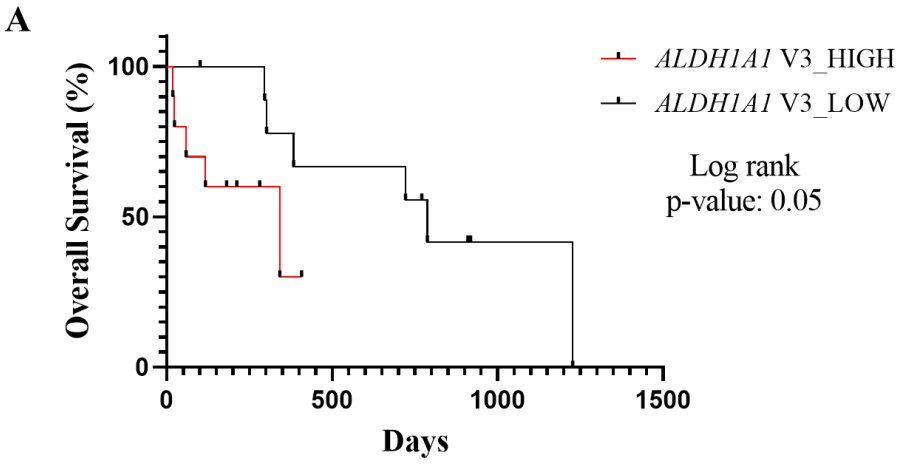
Lastly, to determine the relationship between the aforementioned genes at disease progression, it was performed a contingency analysis. The median values were used as a cutoff to define high/low expression levels. It was observed a positive association of *STAT3* with *PRKCB* (p-value: 0.049), *JAK2* (p-value: 0.005) and *ALDH1A1* (p-value: 0.005). Likewise, there was a positive association between two genes related with apoptotic processes: *BAX* and *CASP8* (p-value: 0.003). Concerning *CCND1-CDK4/6* axis, there was almost a positive association between *CDK4* and *CDK6* (p-value: 0.06). Based on these data, a linear regression analysis was performed to measure the correlation between the above-mentioned genes, but no correlation was found ($R^2 < 0.5$).

A contingency analysis was done to investigate if the gene expression of CTCs at disease progression associated with the clinical characteristics of our patient's cohort (n=20). The median values were used as a cutoff to define high/low expression levels. No significant results were found.

2.6. Identification of prognostic biomarkers at disease progression in CTCs

The CTC gene expression previously analyzed was considered to evaluate the OS in the cohort 1.b. (n= 20) performing a Kaplan-Meier estimator (log-rank test). The median values of CTC gene expression were used as a cutoff to define high/low expression levels.

Higher expression of *ALDH1A1* (342 vs 788 days, p-value: 0.05 by log-rank test) (Figure 23 A) and *STAT3* (296 vs 788days, p-value: 0.02 by log-rank test) (Figure 23 B) associated with shorter OS.



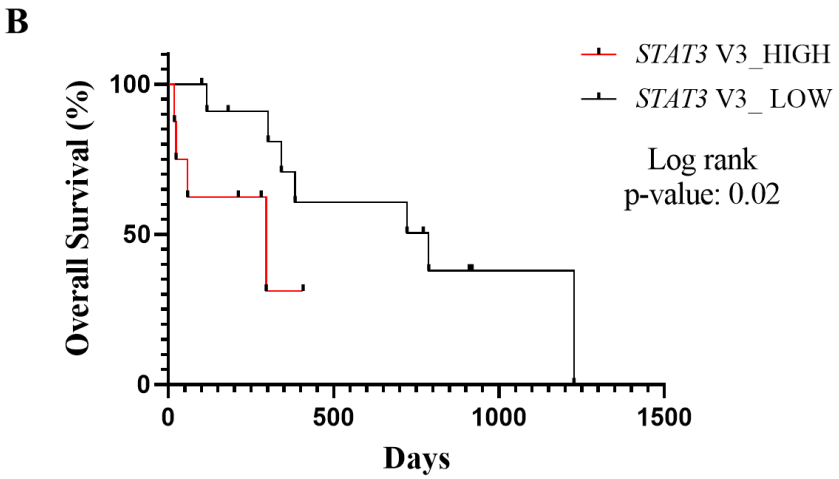


Figure 23. CTC gene expression associated with patient's survival in the cohort 1.b. Kaplan-Meier plot for OS of A) *ALDH1A1* and B) *STAT3* at disease progression. High/low was calculated considering the median CTC expression. Statistics were performed by Log-rank test.

3. CTC STUDY: MUTATIONAL ANALYSIS

3.1. Identification of clinically relevant mutations in CTCs

To identify clinically relevant mutations, a discovery mutational analysis was done by sequencing the CTCs' DNA from 6 HR+/HER2- stage IV BC at visit 1 and 3: 2 *non-responder* and 2 *initial responders* at visit 1 and visit 3, while 2 *long responder* patients at visit 1. A pool of PBMCs from both visits per patient was also analyzed to discard germ-line mutations.

The DNA-seq analysis was performed using a Human Comprehensive Cancer Panel (Qiagen), which identifies alterations in 275 genes. After identifying the mutated genes in our cohort, a GO analysis was performed comparing genes altered on *non-responder* and *responder* patients at visits 1 and 3.

At visit 1, the percentage of genes involved in the PI3K/AKT/mTOR pathway and *CDH1* signaling was higher in *responder* patients (*initial* and *long* responders), while the percentage of genes involved in cell cycle progression was higher in *non-responder* patients (Figure 24).

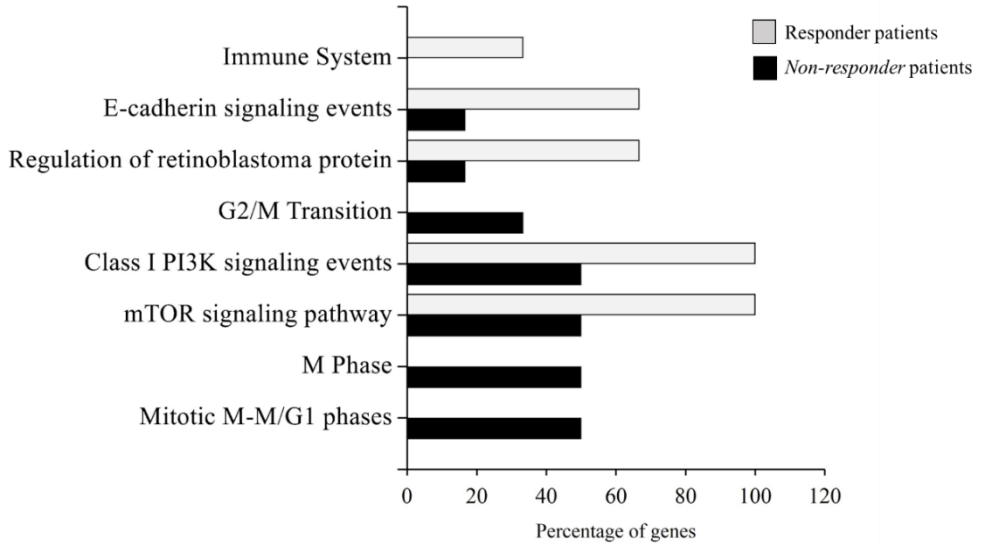


Figure 24. Biological pathways altered in HR+/HER2- stage IV BC patients at visit 1. A GO analysis was performed considering the genes altered in *responder* and *non-responder* patients by DNA-seq using the CTCs' DNA. The germline mutations were subtracted.

At visit 3, the percentage of genes involved in the PI3K/AKT/mTOR pathway and in *CDH1* signaling events was higher in the *non-responder* patients. The percentage of genes related with cell cycle transition from G1 to S phase was higher in the *initial-responder* patients, while the percentage of genes involved in the M phase was higher in the *non-responder* patients (Figure 25).

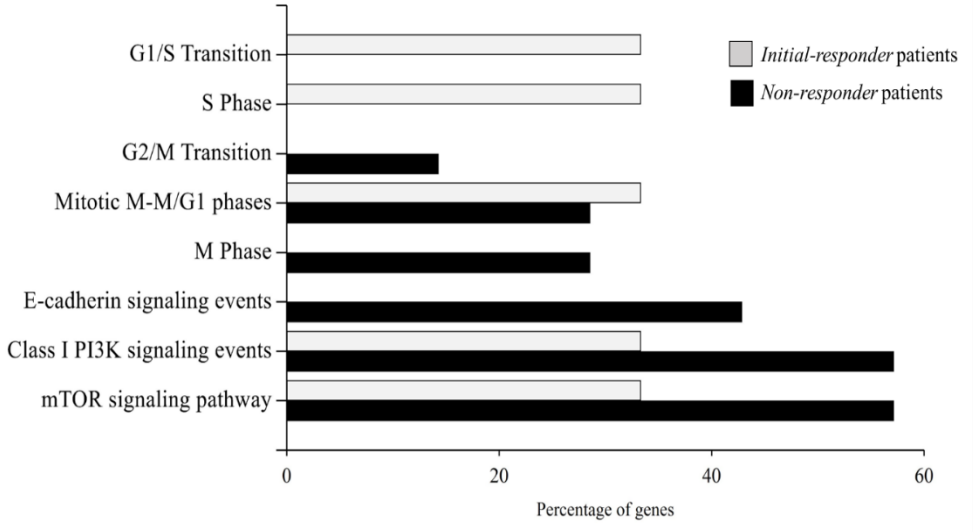


Figure 25. Biological pathways altered in HR+/HER2- stage IV BC patients at visit 3. A GO analysis was performed considering the genes altered in *initial* responder and *non-responder* patients by DNA-seq using the CTCs' DNA. The germline mutations were subtracted.

Later, it was compared the mutations identified in each group at both visit 1 and visit 3 (Table 15). Concerning alterations identified at visit 1, 8 mutations were found in the *non-*

responder group, 5 in the *responder* group while 2 were common in both groups (Figure S7 A). The specific mutations in the *non-responder* group were found in *ARID1A, XPO1, SMO, NOTCH1, SMC3* and *HSP90AA1* genes. All mutations were classified with uncertain significance except for *ARID1A* that was considered as likely benign. The *responder* group had alterations in *ARIND1B, AXIN2* and *EP300*. All patients shared a mutation in *KMT2C* and *C/EBPα* before starting therapy (visit 1). *KMT2C* gene is commonly mutated in BC patients[167,168]. The mutation detected was a pathogenic single-nucleotide variant in an exonic region of *KMT2C* (c.3309T>A, p.C1103*). In *C/EBPα* gene was found an in frame deletion in the promoter region (p.P175del*; p.P224del*; p.P189del*; p.P70del*). At visit 3, 7 mutations were found in the *non-responder* group: *XPO1, BRAF, NOTCH1, SMC3, NOTCH3, AR* and *MED12*. The *initial responder* group had alterations in *MSH6, KMT2C, POLE, FANCA* and *NF1* genes. The last two genes had mutation described as (likely) pathogenic. The rest had benign or uncertain significance. No common mutations were found between *non-responder* and *initial-responder* patients (Figure S7 B).

Table 15. Genes mutated in HR+/HER2- stage IV BC patients detected by CTCs' DNA sequencing at visit 1 and visit 3 (Chr: chromosome, SNV: single number variation)

Gene	Chr	Reference Allele	Sample Allele	Variation Type	Protein variation
ARID1A	1	GCA		Deletion	p.Q1334del
XPO1	2	T	G	SNV	p.K426N
SMO	7	GCT		Deletion	p.L23del
NOTCH1	9	GTG		Deletion	p.P2415del
SMC3	10	G	A	SNV	p.E722K
HSP90AA1	14	CCT		Deletion	p.E16del;
					p.E138del
ARID1B	6	TCC		Deletion	p.S41del
					p.124del
AXIN2	17	TGG		Deletion	p.H474del
EP300	22	CAG		Deletion	p.Q2199del; p.Q2225del
KMT2C	7	A	T	SNV	p.C1103
C/EBPa	19	TGG		Deletion	p.H474del
BRAF	10	C	T	SNV	p.V587I; p.V642I; p.V679I; p.V602I; p.V605I; p.V639I; p.V617I
NOTCH1	9	GTG		Deletion	p.P2415del
NOTCH3	19	CA		Deletion	p.C720*
AR	X		GCA	Insertion	p.Q80dup
		GGCGGCGGCGGCGGCGGC		Deletion	
MED12	X	GCA		Deletion	p.S655del
MSH6	2	C	A	SNV	p.R62R
POLE	12	A		Deletion	p.G468_G473del
FANCA	16	AG		Deletion	p.W911fs*31
NF1	17	T	G	SNV	p.S340A

3.2. Analysis of CTCs' mutations in the ctDNA

Four mutations identified in the CTCs' DNA from *non-responder* patients were selected for further investigation in the ctDNA based on their potential significance as a biomarker of therapy response (*AR*, *MED12*, *SMC3* and *HSP0AA1*). The ctDNA from 10 HR+/HER2- stage

IV BC patients from cohort 1: 4 *non-responder* and 6 *responder* patients (3 *initial* and 3 *long* responder) was analyzed by ddPCR. The probes designed for *AR* and *MED12* did not recognized the specific alteration due to the repetitive sequences, then, the probes were discarded for the validation step. Besides, the results obtained for *SMC3* were also discarded because the wildtype probe detects the alteration, pointing to unspecific amplification. Concerning *HSP90AA1* mutation, the alteration was identified in the ctDNA from all patients analyzed. No differences in the number of events were found among groups (Figure 26).

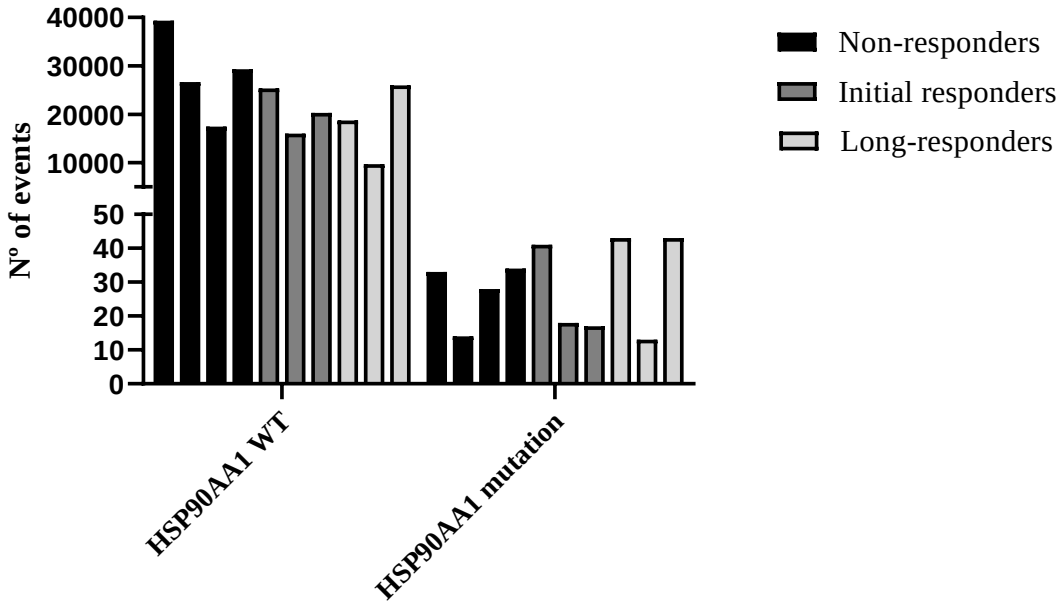


Figure 216. Nº of events with *HSP90AA1* wild type or mutated in the ctDNA from 10 HR+/HER2- stage IV BC patients: 4 *non-responder*, 3 *initial responder* and 3 *long responder* patients. The ctDNA was analyzed by ddPCR after a preamplification process.

3.3. *PI3K* mutation analysis in the ctDNA from HR+/HER2- stage IV BC patients

Several trials had described that alterations in *PI3KCA* gene can reduce sensitivity to CDK4/6i plus ET. Based on SOLAR-1 trial, clinical guidelines recommend that HR+/HER2- BC patients with local recurrence or metastatic progression should be tested for PIK3CA mutations using NGS in tumor tissue or ctDNA. Thus, the presence of the most common PI3K mutations (p.E542K, p.E545K, p.H1047L and p.H1047R) was studied in the ctDNA from cohort 1 (n= 17 in cohort 1.a. at visit 1 and n=21 in cohort 1.a. at visit 3). A total of 4 patients had *PI3K* mutated at basal (visit 1), while 10 at progression (visit 3). 9 out of 10 patients that had *PI3K* mutated were *responders* (3 *initial responders* and 5 *long responders)*. No association between the *PI3K* mutational status and survival was detected (p-value: 0.7).

Considering the CTC gene expression and the presence of *PI3K* alterations in the ctDNA from paired samples of HR+/HER2- stage IV BC patients, it was investigated whether the CTC expression is related to the PI3K pathway. No association was found before starting therapy. However, patients showing *PI3K* mutated at progression had a higher expression of *HDAC6*

(p-value: 0.03) (Figure 27 A), while lower expression of *BAX* (p-value: 0.04), *CASP8* (p-value: 0.029), *CUL1* (p-value: 0.023) and *RAC2* (p-value: 0.017) (Figure 27 B-E).

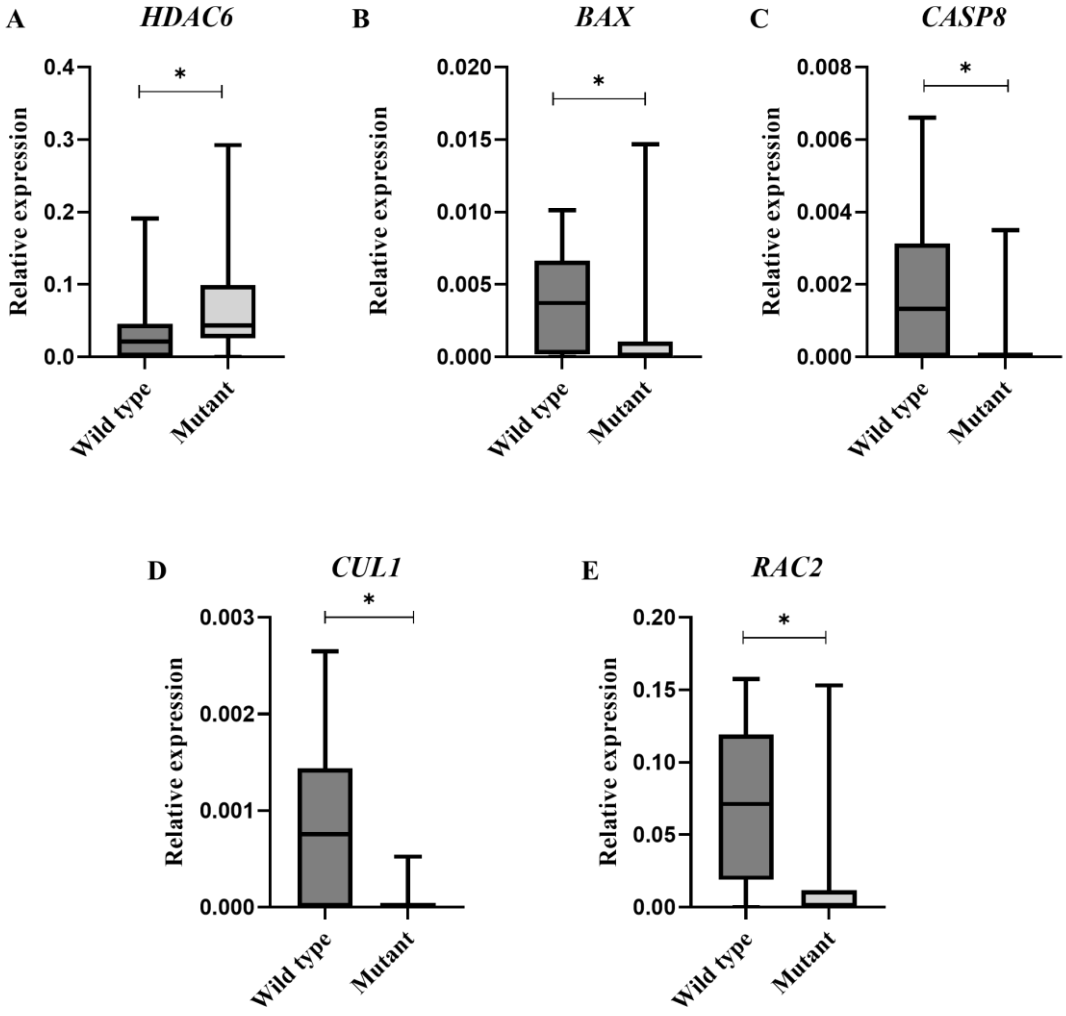


Figure 27. *PI3K* mutation associated with CTC expression at disease progression (n=20). A) *HDAC6*, B) *BAX*, C) *CASP8*, D) *CUL1* and E) *RAC2*. The CTC expression was analyzed by RT-qPCR and normalized to *B2M* and autologous PBMCs. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***).

4. LONGITUDINAL STUDY OF cfDNA

cfDNA levels are widely used to determine patients' prognosis. In this study it was isolated the cfDNA from 20 stage IV HR+/HER2- BC patients at different time points: visit 1, visit 2, visit 3 and every 3 months since visit 1 up to 12 or 24 months. It was not possible to obtain the blood from all time points in the whole cohort.

It was longitudinally studied the cfDNA levels in the above-mentioned time points to ahead disease progression. It was observed that the cfDNA levels vary along the disease, but cfDNA sampling every 3 months is not enough to ahead of disease progression in HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET (Figure S8). Moreover, the cfDNA concentration

was not statistically different among visits or groups (based on therapy response) in neither visit (Figure S9 and S10).

As the cfDNA concentration *per se* did not determine therapy response accurately, a cfDNA ratio (CDR) was calculated comparing the concentration at 3 and 6 months with visit 1 in 10 HR+/HER2- stage IV BC patients from cohort 1 (see section 4.3.1., M&M). It was defined that CDR<1 is a reduction in cfDNA concentration, CDR=1 no changes in cfDNA concentration while CDR>1 is an increase in cfDNA concentration. In the *responder* group, CDR<1 was found both at 3 (p-value: 0.05) and 6 months (p-v+alue: 0.01) (Figure 28 A-B). Next, using CDR<1 as a cut-off, a survival analysis was performed. CDR<1 associated with shorter PFS at 3 months (122.5 days vs 430.5 days, p-value: 0.03 by log-rank test) (Figure 28 C) and 6 months (105 days vs 517 days, p-value: 0.01 by log-rank test) (Figure 28 D). No association was found between CDR or cfDNA levels and CTC gene expression or clinical variables.

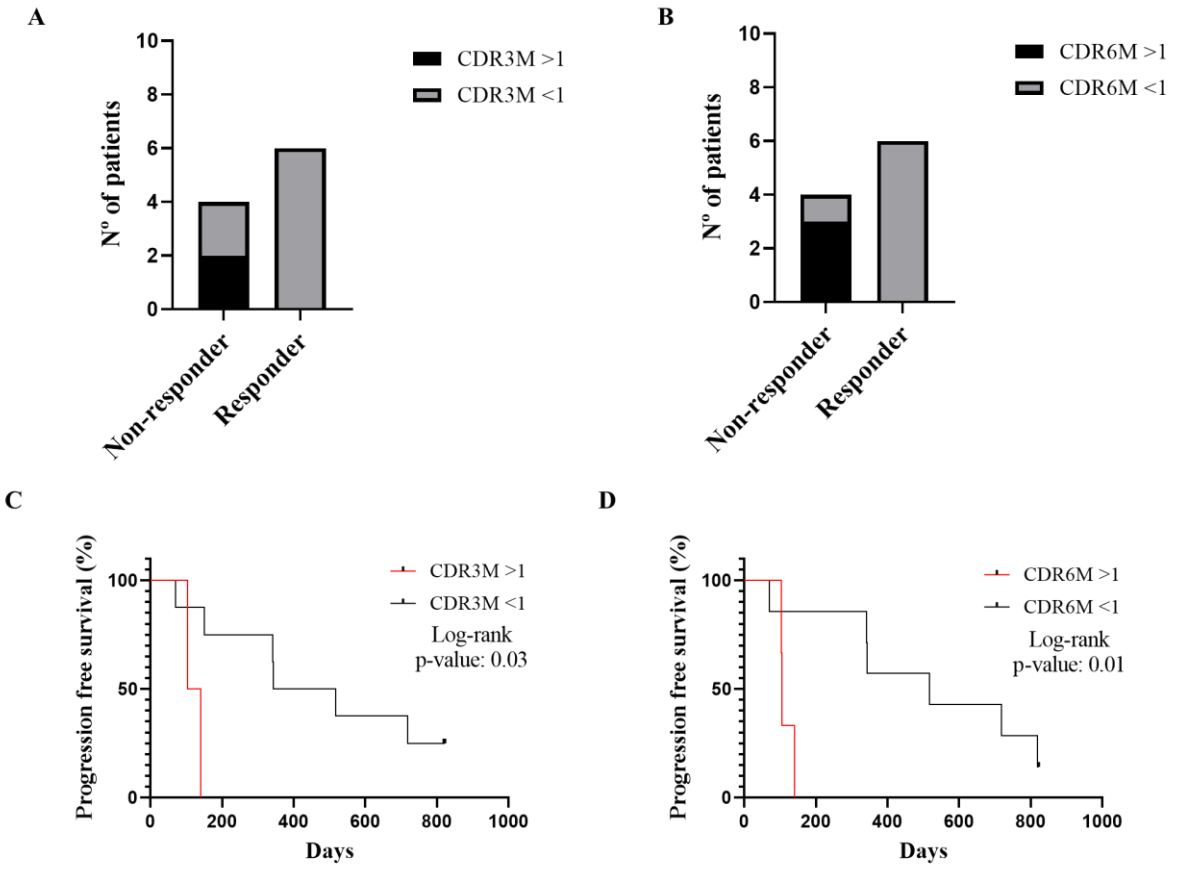


Figure 28. The CDR determined therapy response and patient's outcome. Percentage of patients showing A) CDR3M > 1 and B) CDR6M > 1. Kaplan-Meier plot for PFS of C) CDR3M and D) CDR6M. Statistics were performed by Fisher's exact test and Log-rank test.

Next, to calculate a threshold to predict patient's response to polytherapy considering cfDNA concentration and CDR, a ROC analysis was performed. The best AUC was obtained at 6 months in both approaches, 0.95 (Figure 29 A) and 0.87 (Figure 29 B), respectively.

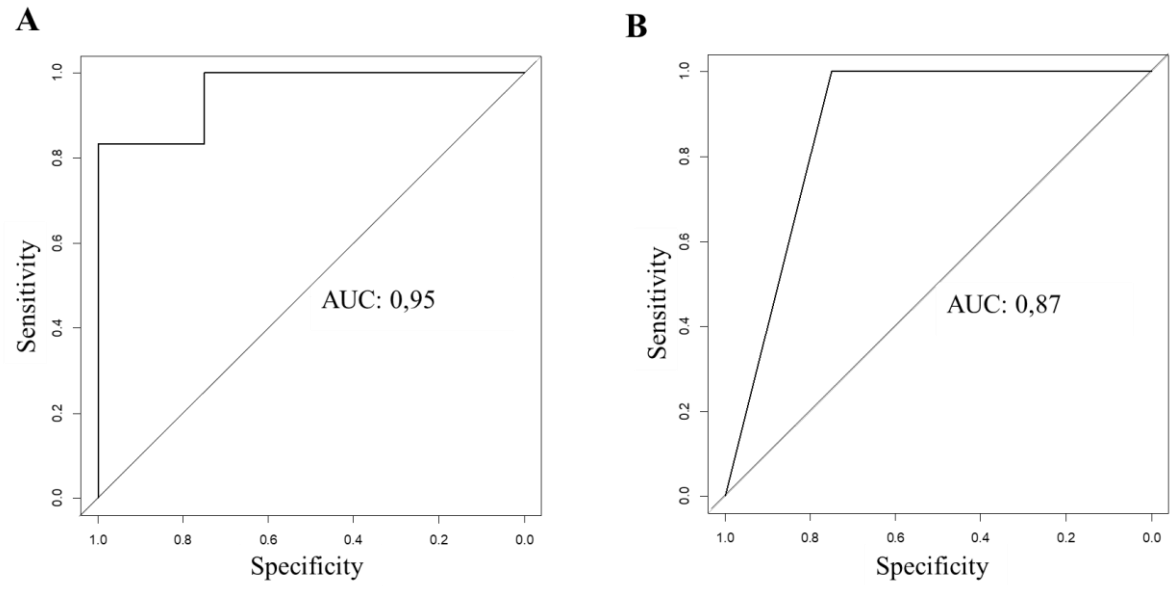


Figure 29. ROC analysis to classify patients according to therapy response considering A) cfDNA concentration at 6 months and B) the CDR6M. (CDR: cell-free DNA ratio).

5. INFORMATIVE BIOMARKERS IN PBMCs

The immune cells are an integral component of tumor microenvironment. It was proposed that the crosstalk between cancer and immune cells transforms the immune fraction. Then, one objective of the current study is to identify the PBMCs-associated biomarkers with clinical relevance. For that, PBMCs from peripheral blood were isolated with Lymphoprep. Moreover, as the leukocyte count tends to downturned in various malignancies, the blood test data was taken into account in those patients for whom the data was available.

5.1. Identification of predictive biomarkers in PBMCs

As PBMCs samples were also included in the gene discovery study performed for the CTCs' biomarker identification (PanCancer Pathway Panel with nCounter technology, M&M), it was studied if the transcriptome of PBMCs also determines therapy response. For that, it was analysed the PBMCs' RNA from 6 HR+/HER2- stage IV BC patients (2 non-responder, 2 initial-responder and 2 long-responder patients) as a pool of samples from visit 1 and 2. Then, a DGE analysis was done using the DESEq2 package to determine which genes were differentially expressed among groups. The long responder patients were used as the control group.

A total of 505 genes were differentially expressed in the PBMC samples: 158 downregulated and 347 upregulated in the *non-responder* compared with *responder* patients (Figure 30).

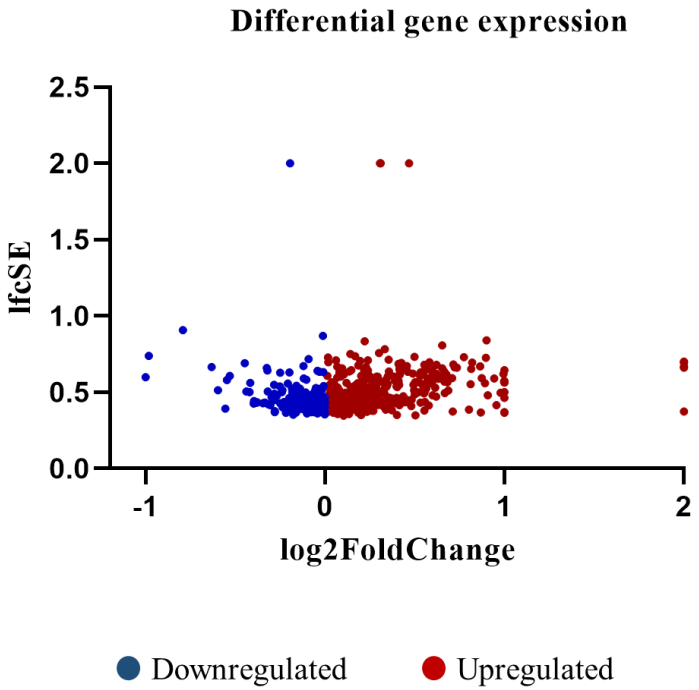


Figure 30. Volcano plot of PBMCs' RNA counts after DGE analysis, using the *long* responder patients as a reference group. Downregulated genes are depicted in blue while upregulated genes in red. Statistics were performed by the DESEq2 package.

Based on FDR and p-value, 12 genes were selected for validation by RT-qPCR in 26 stage IV HR+/HER2- BC patients in order to identify predictive biomarkers of polytherapy response (Table S1). PBMC gene expression was relativized to *TGFBR2* expression.

Then, the gene expression of PBMCs was compared between *non-responder* and *responder* patients. It was found that *GZMB* expression is significantly lower in *non-responder* compared with *responder* stage IV BC patients (p-value=0.04) (Figure 31 A). In *responder* patients, *KLF4* expression was higher compared with *non-responders* (p-value: 0.02) (Figure 31 B). In addition, *MYCL* had a significantly lower expression in *non-responder* patients compared with *responder* stage IV patients (p-value= 0.009) (Figure 31 C).

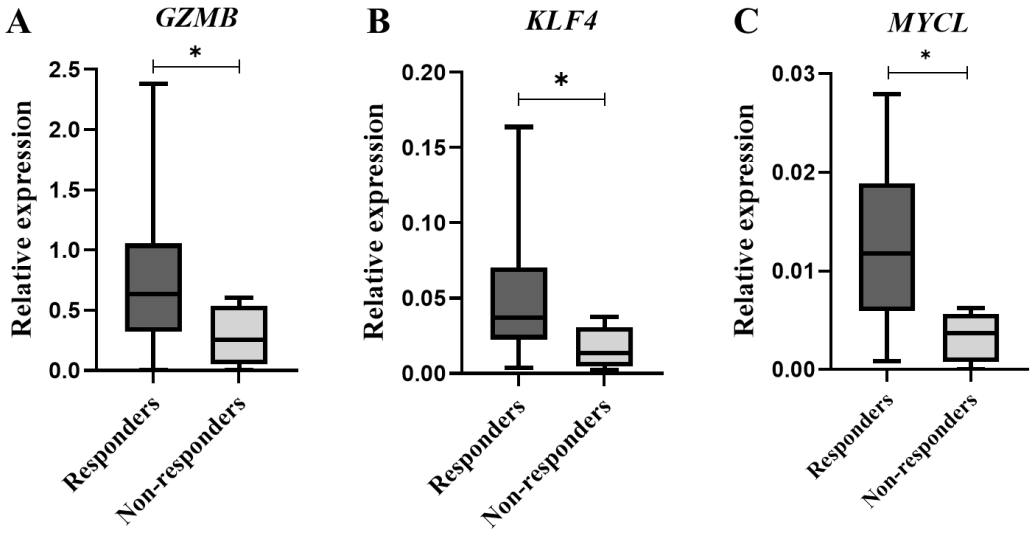


Figure 31. PBMC gene expression from *non-responder* (n=5) and *responder* patients (n=21) before starting therapy. A) *GZMB*, *B) KLF4* and C) *MYCL*. The expression was analyzed by RT-qPCR and normalized to *TGFBR2*. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

5.2. Identification of prognostic biomarkers in PBMCs

As PBMC gene expression predicts therapy response in HR+/HER2- stage IV BC patients, it was also investigated whether the expression of the analyzed genes is altered in BC regardless of the stage. For that, the expression of the aforementioned analysed genes was also studied in 20 cancer-free women and 21 HR+ stage I-III BC patients. *GZMB* did not differ among groups (p-value>0.05). *KITLG, KMT2D* and *KLF4* showed a higher expression in cancer-free women compared with stage I-III (p-value: 0.02; 0.001; 0.0008, respectively) and stage IV (p-value: 0.0002; 0.02; 0.001, respectively) BC patients (Figure 32 A-C). Besides, *KITLG* expression in stage I-III patients was higher than in stage IV patients (p-value: 0.03). It was noticed that *KITLG* expression decrease according to disease stage (Figure 32 A). Lastly, the expression of *MYCL* was significantly lower in the *non-responde*r patients compared with the other groups (Figure 32 D).

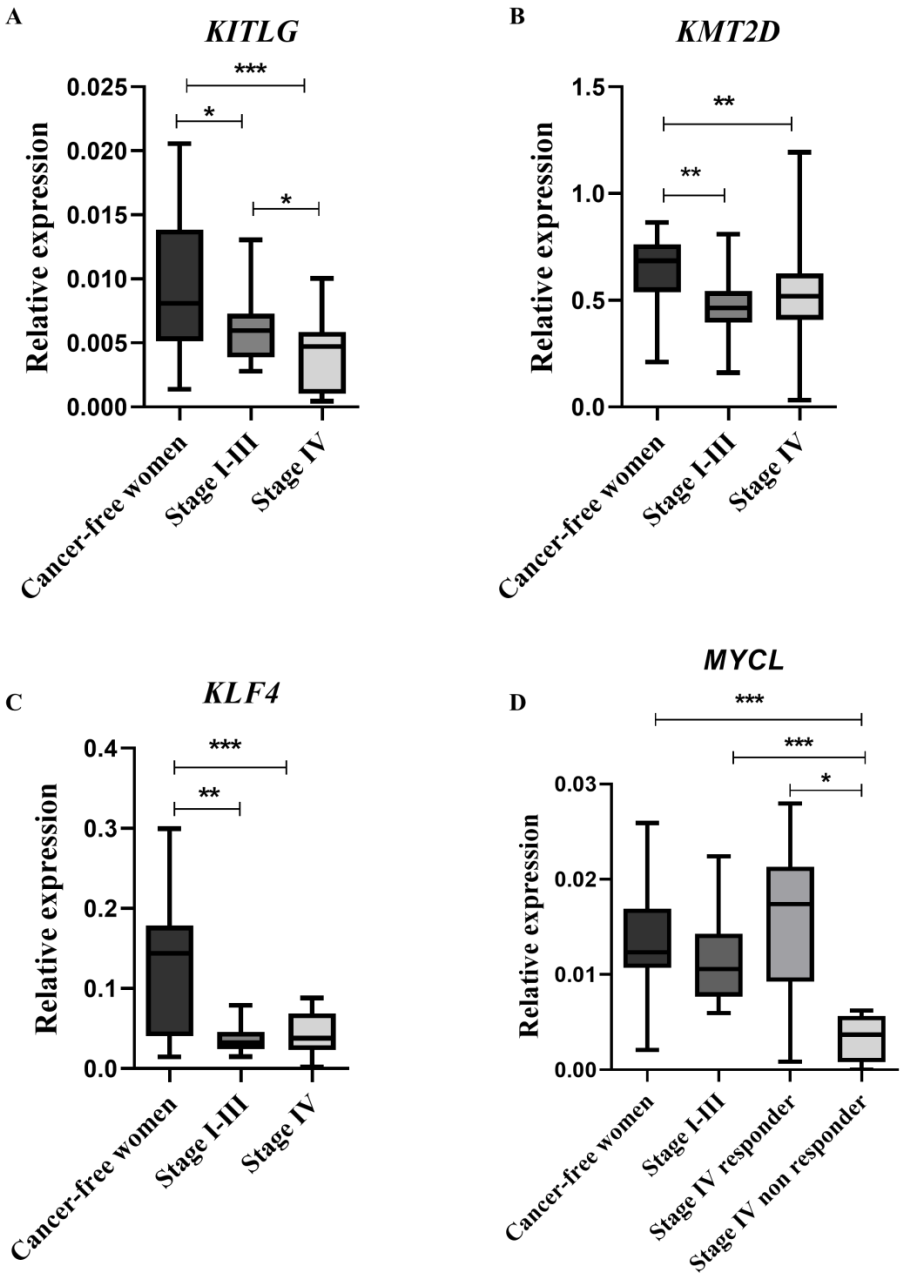


Figure 32. PBMC gene expression from cancer free-women (n=20) and cancer patients (n=47) before starting therapy in HR+ subtype. A) *KITLG*, *B) KMT2D* and C) *KLF4* and D) *MYCL*. The expression was analyzed by RT-qPCR and normalized to *TGFBR2*. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

Afterward, it was determined if the previously described PBMCs expression changes are not only dependent of BC stage but also of cancer subtype. Thus, the expression of *GZMB*, *KITLG*, *KMT2D*, *KLF4* and *MYCL* was analyzed in PBMCs from different TNBC stages and compared with the HR+ subtype samples. Firstly, the gene expression of PBMCs was compared among 20 cancer-free women, 13 stage I-III and 18 stage IV TNBC patients. *GZMB* and *KMT2D* expression did not differ among stages (p-value > 0.05). *KITLG* showed a higher expression in cancer-free women compared with stage I-III (p-value: 0.04) and stage IV (p-

value: 0.05) TNBC patients, but no differences were found among BC stages (Figure 33 A). *KLF4* had a higher expression on cancer-free women compare with stage I-III (p-value: 0.0008) and stage IV (p-value: 0.003) patients (Figure 33 B). Lastly, the expression of *MYC-L* was higher in cancer-free women than in stage IV TNBC patients (p-value: 0.004). The expression in the stage I-III is also higher, but the result was not significant (p-value: 0.06) (Figure 33 C). Then, PBMCs gene expression from stage I-IV BC patients was compare between HR+ and TNBC subtypes, but not differences were found (p-value > 0.05).

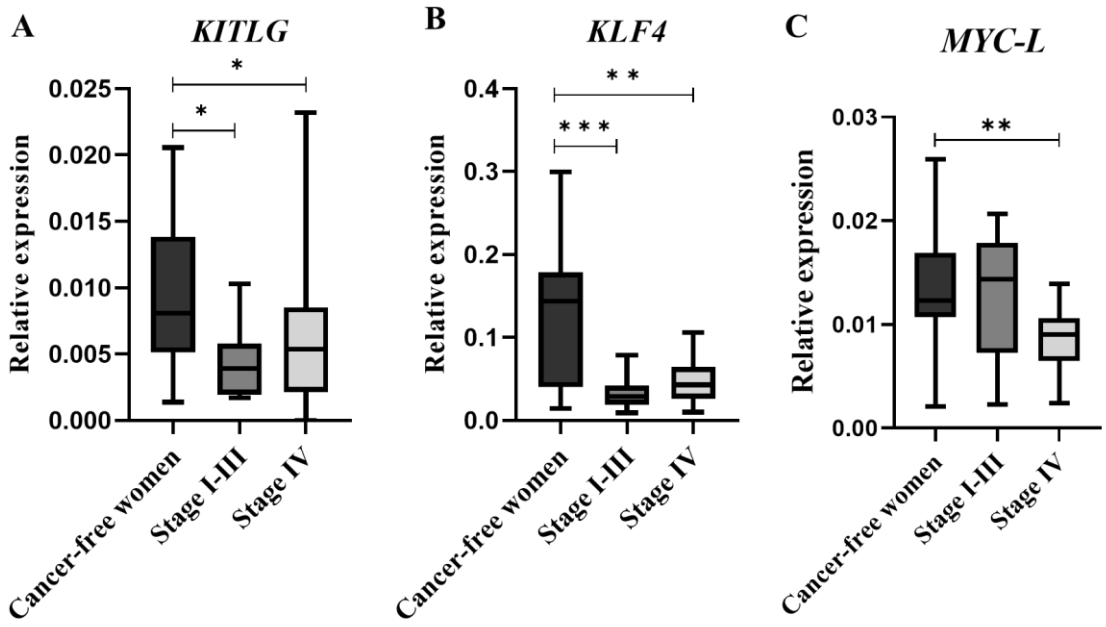


Figure 33. PBMC gene expression from cancer free-women (n=20) and cancer patients (n=31) before starting therapy in the TNBC subtype. A) *KITLG*, *B) KLF4* and C) *MYCL*. The expression was analyzed by RT-qPCR and normalized to *TGFBR2*. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

5.3. *GZMB* as a predictive biomarker

Previously, it was observed that *GZMB* expression associated with therapy response both in the CTC-enriched fraction and in PBMCs. A deeply analysis of this genes was performed because it seems as a potential prognostic biomarker, but also there is an unmet need to decipher if the protein is internalized in tumor cells or exist non-depleted PBMCs in the CTC-enriched fraction. For that, the *GZMB* protein expression was measured by flow cytometer in PBMCs from 8 HR+/HER2- stage IV BC patients (4 *non-responder* and 4 *responder* patients) from cohort 1 to determine if GZMB percentage also informed about therapy response. The mean percentage of GZMB+/PTPRC+ cells was 34.9 % in the *non-responder* patients, while 44 % for *responder* patients. No statistical difference was found between groups. Moreover, GZMB+/PTPRC+ was analyzed in PBMCs from 3 stage I-III BC patients to determine if the *GZMB* secretion depends on the BC stage. It was observed higher GZMB+/PTPRC+ levels in stage IV BC patients (p-value:0.04) than in early BC stages. Lastly, the percentage of

GZMB+/PTPRC+ cells in the CTC enriched fraction from 2 stage IV BC patients was measured to determine if the GZMB+ immune cell subpopulation was enriched after RosetteSep. It was observed a higher percentage of GZMB+/PTPRC+ cells in the CTC enriched fraction than in the PBMC fraction, but the difference was not statistically significant.

Based on the results obtained analyzing the *GZMB* expression in the CTC-enriched fraction and in PBMCs, *GZMB* expression could be a response biomarker. To check it out, a ROC analysis was performed using the *GZMB* expression from the CTC-enriched fraction and from PBMCs to distinguish responder patients. The AUC curve obtained in the enriched fraction of CTCs classified correctly 17 out of 21 (81 %) patients with an AUC=0.79 (Figure 34 A). The AUC curve obtained from PBMCs classified all patients (100 %) accordingly to therapy response, AUC=1 (Figure 34 B).

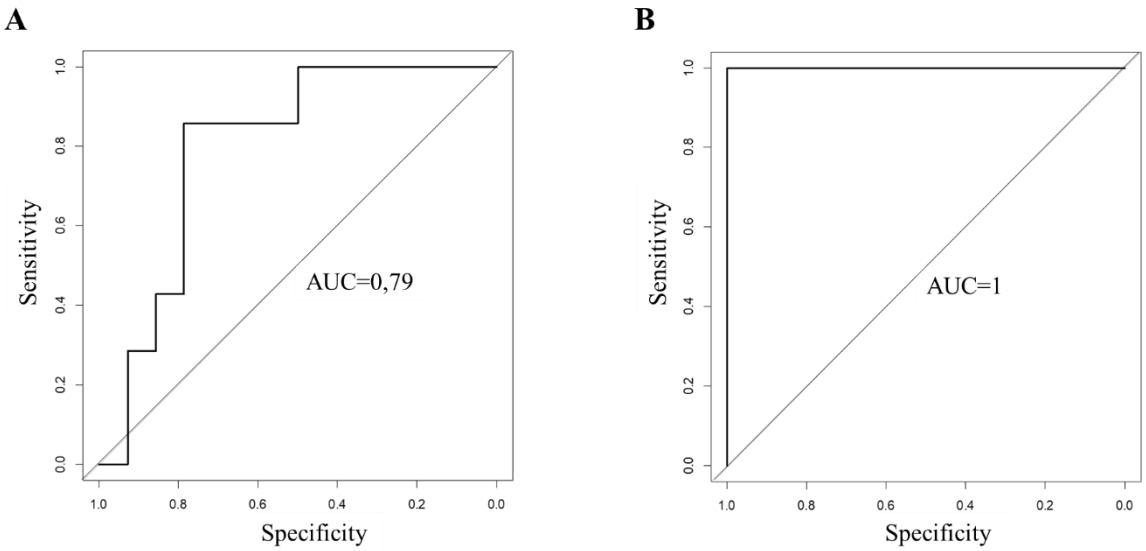


Figure 34. ROC analysis considering the *GZMB* gene expression A) in the CTC-enriched fraction (n=21) and B) in PBMCs (n=26) at visit 1.

6. WHITE BLOOD CELLS COUNT

6.1. Changes in leukocyte levels in stage IV breast cancer patients

In order to confirm if the leukocyte count is altered in the advanced disease, blood test data from 28 HR+/HER2- stage IV BC patients was studied both at visit 1 and 2. After one-cycle therapy, there was a decreased in the number of leukocytes compare with before starting therapy (neutrophils, p-value: $7x10^{8}$; lymphocytes, p-value: 0.05; monocytes, p-value: 0.00003 and eosinophils, p-value: 0.00013) (Figure 35 A). Besides, the leukocyte type whose levels changed varies depending on therapy response (Figure 35 B-D). Firstly, in the *long responder* group (n=9) was observed a reduction of neutrophils (p-value: 0.0016) and eosinophils (p-value: 0.002) levels (Figure 35 B). In the *initial responder* group (n=14), there was a reduction of neutrophils (p-value: 0.00007) and monocytes (p-value: 0.007) levels (Figure 35 C). Lastly, in

the *non-responder* group (n=5) there was a reduction in monocytes levels (p-value: 0.007) (Figure 35 D).

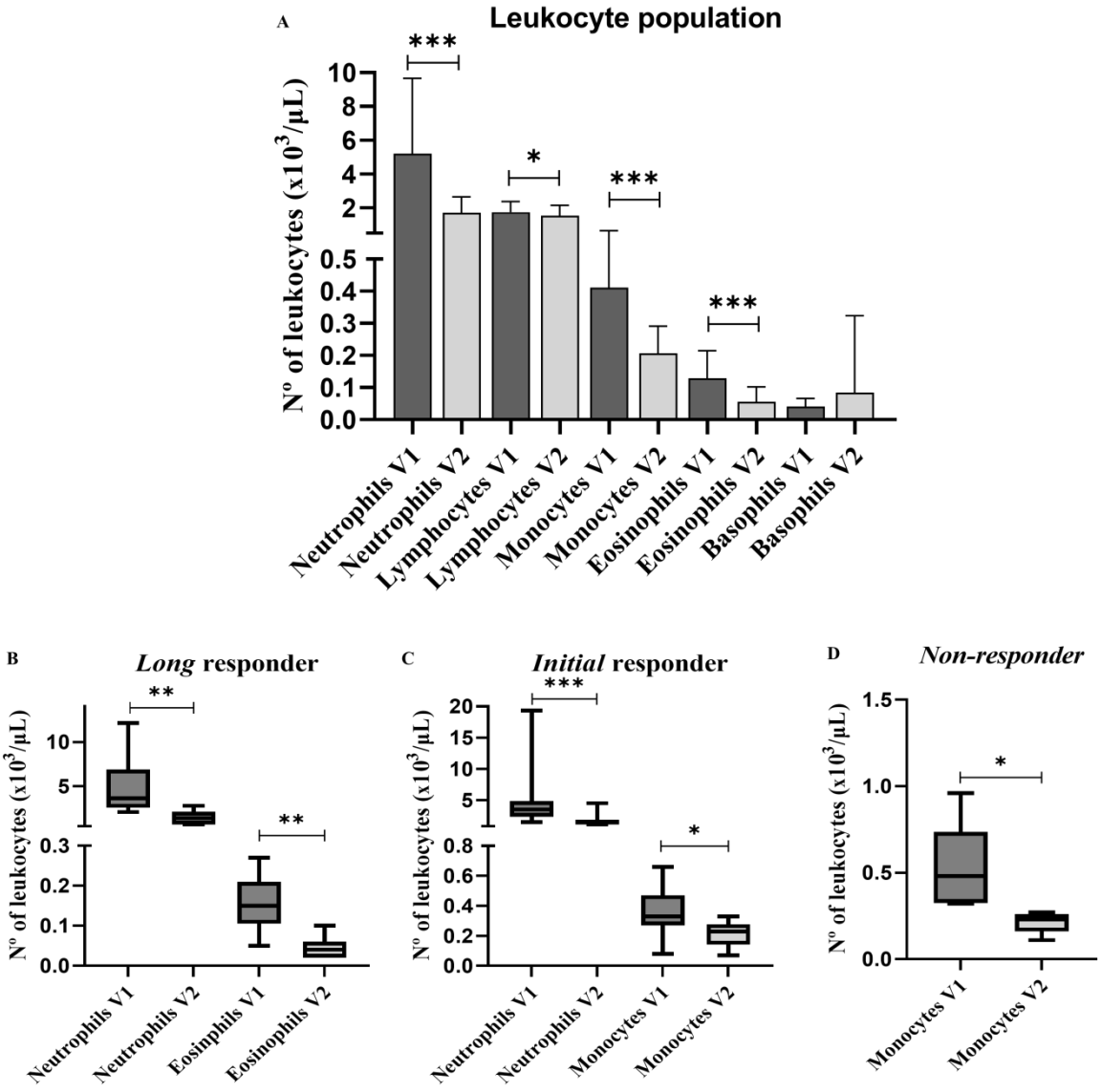


Figure 35. Leukocyte population counts. A) Leukocyte counts before starting therapy (visit 1) and after one-cycle therapy (visit 2), B) Different count of leukocytes in *long* responder at visit 1 and 2, C) Different count of leukocytes in *initial* responder patients at visit 1 and 2 and D) Different count of leukocytes in *non-responder* patients at visit 1 and 2. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

In order to confirm if the observed alteration in leukocyte levels were dependent on BC subtype, it was studied the leukocyte count in 19 HR+/HER2- stage IV TNBC patients both at visit 1 and 2. There was a decrease in the number of leukocytes after one-cycle therapy (neutrophils, p-value: 0.003; lymphocytes, p-value: 0.003 and eosinophils, p-value: 0.003) except for monocytes and basophils (Figure 36).

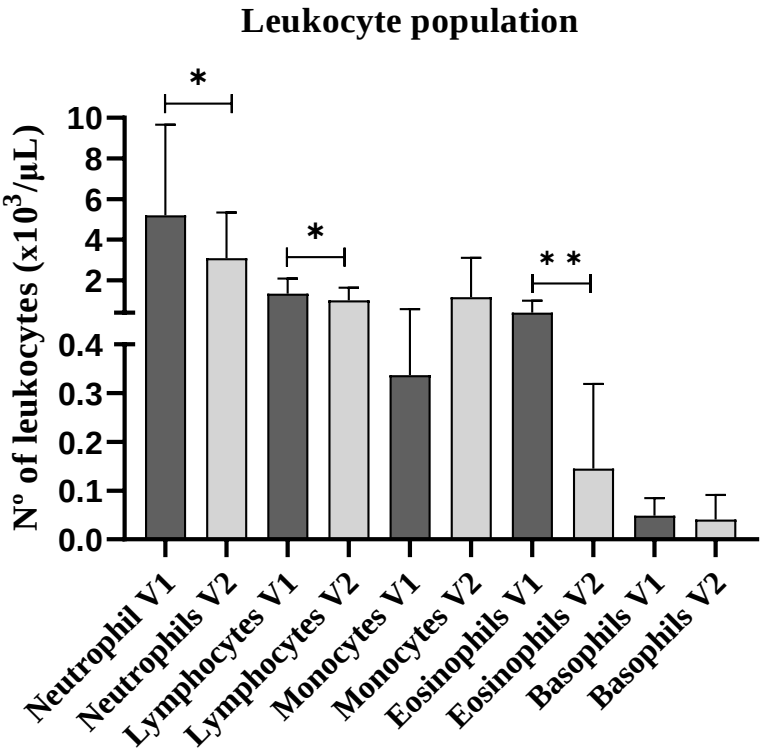


Figure 36. Leukocyte population counts at visit 1 and 2 in stage IV TNBC patients. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***).

Comparing the number of leukocytes between HR+/HER2- and TN BC stage IV subtypes after one-cycle therapy, there was a difference in neutrophils (p-value: 0.02), lymphocytes (p-value: 0.03), monocytes (p-value: 0.0002) and eosinophils (p-value: 0.029) levels (Figure 37).

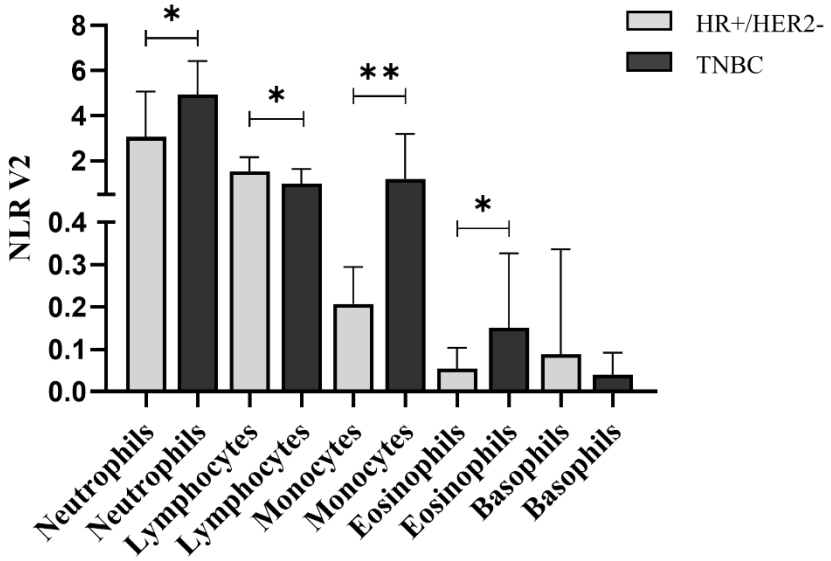


Figure 37. Leukocyte population levels in HR+/HER2- and TN stage IV BC patients after one-cycle therapy. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***).

The NLR, a ratio between neutrophil and lymphocyte counts in peripheral blood, is an emerging marker of immune response. A value of NLR = 1-3 implied a normal physiologic stress level, NLR = 6-8 a mild physiologic stress levels, NLR = 9-18 moderate physiologic stress levels and NLR > 18 a severe physiologic stress level. In our cohort of HR+/HER2- and TNBC patients, the NLR did not correlate with patient's clinical data, response to therapy, survival or expression, although the NLR differs between subtypes both at VISIT 1 and 2 (Figure 38 A-B). Additionally, the subtypes also differed in the physiological stress conditions at both visits. After one-cycle therapy, all HR+-HER2- stage IV BC patients had a normal NLR, while in the TNBC there are patients with a severe physiological stress (Figure 38 C-D). No differences were found between non-responder and responder patients in the HR+/HER2- stage IV BC subtype.

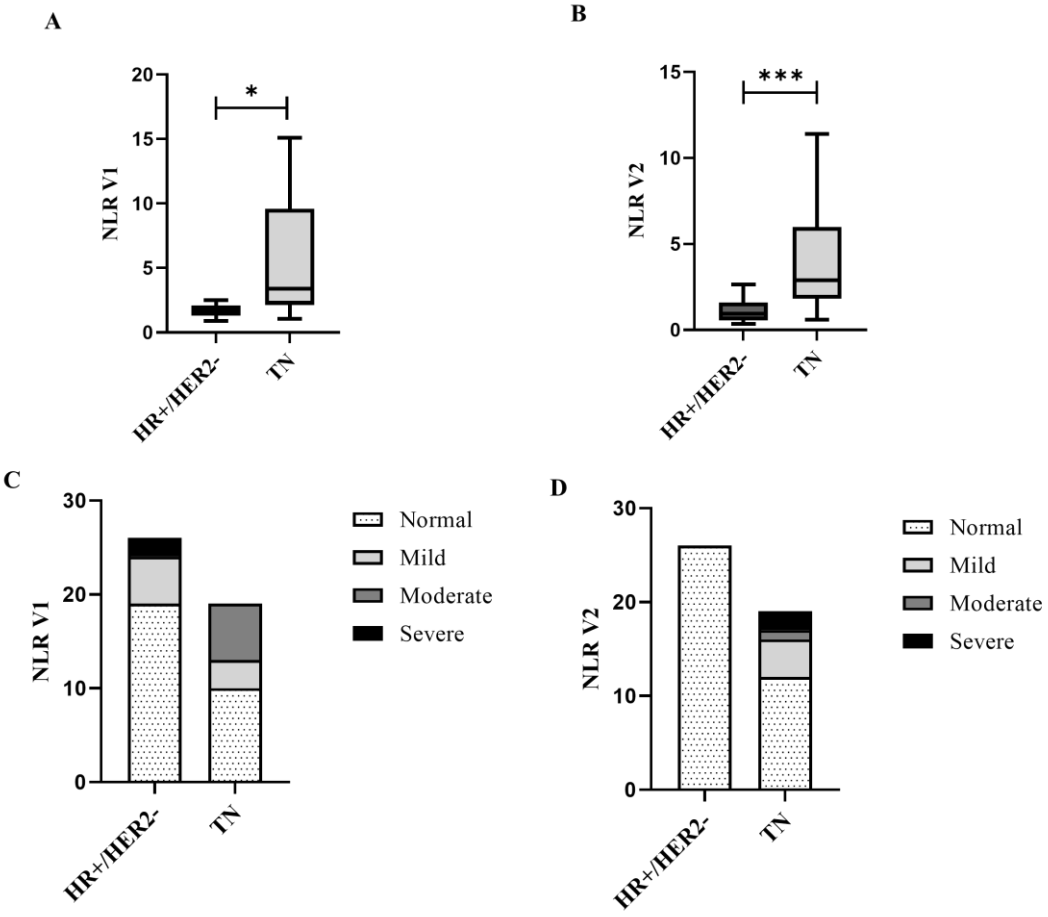


Figure38. NLR in HR+/HER2- and TNBC subtype. There was a difference in the mean value of NLR between subtypes (A-B) as well as in the physiological stress conditions (C-D) at visit 1 and 2. Statistics were performed by Wilcoxon and Fisher's exact test (p-value < 0.05 (*), < 0.01 (**) and < 0.001(***).

7. PBMCs AND CELL LINES INTERACTION

Taking into account the crosstalk between the immune system and cancer cells, it was determined the PBMCs' and BC cells transcriptional changes when both cell types were co-

cultured together. For that, MCF7 human derived BC cell line ($2x10^5$ cells) and PBMCs freshly isolated from cancer-free women (n=8, $5x10^6$ cells), stage I-III (n=8, $5x10^6$ cells) and stage IV (n=8, $2x10^6$ cells) BC patients were used to establish an *in vitro* paracrine co-culture (CC). Then, a gene expression analysis was made on both PBMCs and MCF7.

7.1. Effects of MCF7 cell line on PBMCs' gene expression

The genes previously identified as differentially expressed in PBMCs (section 5.1., Results) were analysed in the immune cells after paracrine CC with MCF7 tumor cells, to determine how cancer cells influence on PBMC gene expression. After paracrine CC, it was observed a reduction in *KITLG* (p-value: 0.039) (Figure 39 A) and *KLF4* (p-value: 0.0390) (Figure 39 C) levels in cancer-free women PBMCs, as well as *KITLG* (p-value: 0.015) (Figure 39 A) and *KMT2D* (p-value: 0.039) (Figure 39 B) in stage I-III patients PBMCs. Although there was a decreased in all genes analysed, no significant changes were detected in PBMCs from stage IV patients after CC.

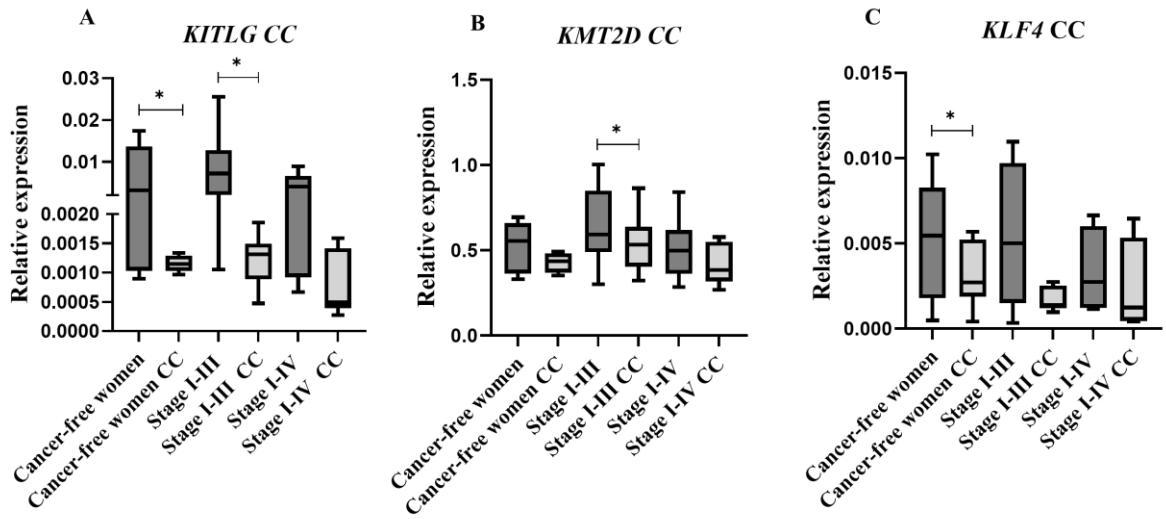


Figure 39. PBMC gene expression after culturing with MCF7 BC cell line. A) *KITLG*, B) *KMT2D* and C) *KLF4*. PBMC expression was normalized to *TGFBR2*. Statistic was performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

7.2. Effects of PBMCs on MCF7 cell line gene expression

Next, the gene expression of MCF7 cell line was assessed after co-culturing with PBMCs from cancer-free women and stage I-IV BC patients to determine how PBMCs from different BC stages influence on cancer cell gene expression. For that, the expression of the proliferative marker (*KI67*), apoptosis-related markers (*caspases 8* and *9*) and an epithelial marker (*CDH1)* were studied in MCF7 cell line.

A significant decrease in *KI67* expression was observed in MCF7 cells CC with PBMCs from cancer-free women (p-value: 0.003) and stage I-III (p-value: 0.015) patients. The *KI67*

reduced levels wre also observed in PBMCs froms stage IV BC patients, but it was not statistically significant (p-value: 0.09) (Figure 40 A). It was also observed a statistical difference in *CASP8* expression when MCF7 was CC with PBMC from stage IV patients (p-value: 0.03) (Figure 40 B). However, no difference in *CASP9* expression was observed (data not shown). The expression of *CDH1* was reduced when MCF7 cell were CC with PBMCs from cancer free women (p-value: 0.03), stage I-II (p-value: 0.05) and stage IV (p-value: 0.003) BC patients (Figure 40 C).

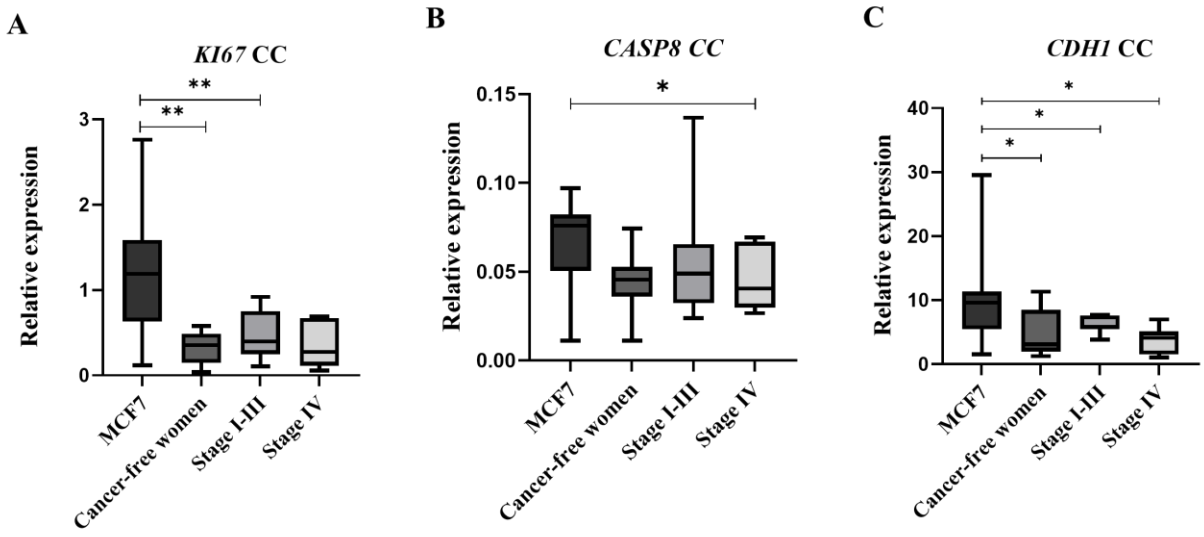


Figure 40. MCF7 cell line gene expression after CC with PBMC from cancer free women and BC from different stages. A) *KI67*, B) *CASP8* and C) *CDH1*. MCF7 expression was normalized to *B2M*. Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

8. CDK4/6i RESISTANT TUMOR CELL LINES

8.1. Generation and characterization of CDK4/6i resistant tumor cell lines

To generate HR+/HER2- tumor cell lines resistant to CDK4/6i, MCF7 estrogen-dependent human breast cancer derived cell line was treated with Palbociclib, while T47D progesterone-dependent human breast cancer derived cell line was treated with Ribociclib. Initially both cell lines were treated with CDK4/6i's IC50 for 2 weeks (0.2 µM for Pablociclib and 4 µM for Ribociclib). Then, the double IC50 concentration was used (section 13.2., M&M). Cell cycle retention in the G1 phase was checked by flow cytometer MCF7 Palbociclib resistant (MCF7p-R), T47D Ribociclib resistant (T47Dr-R) and in their counterparts (Figure 41). Once confirmed the cell cycle retention in the phase G1, it was carried out both proliferation and colony formation assays as well as molecular characterization by RT-qPCR.

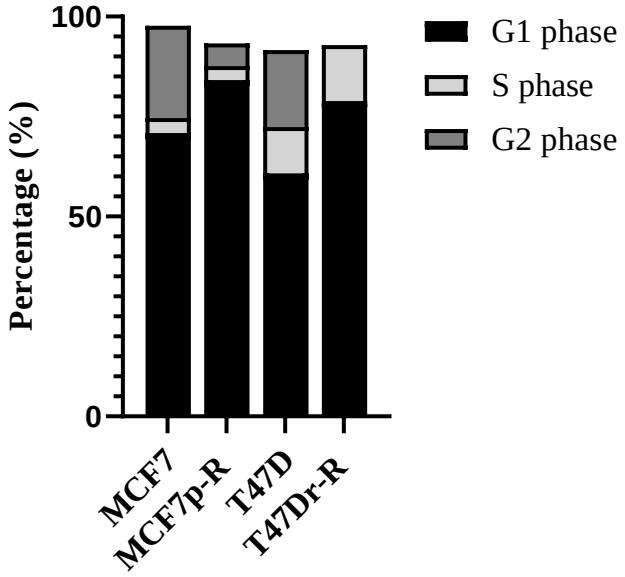


Figure 41. Percentage of MCF7 and T47D cells retained in each cell cycle phase after treatment with one dose of the IC50 of Palbociclib and Ribociclib, respectively. MCF7 (G1: 70, S: 3; G2: 23); MCF7p-R (G1: 84, S: 3; G2: 5); T47D (G1: 60, S: 11; G2: 19); T47Dr-R (G1: 78, S: 14; G2: 0)

The MCF7p-R, T47Dr-R cell lines and their parental counterparts were cultured with the IC50 of the CDK4/6i or fresh-DMEM for a week to determine the resistance acquisition.

Once the CDK4/6i was added, the proliferation was reduced in both MCF7p-R and MCF7 parental cell line a 25 % and a 47 %, respectively. That difference was not significant (Figure 41 A). However, MCF7p-R growth more than its counterpart when both cells were cultured with fresh-DMEM (p-value: 0.002) (Figure 42 A).

Concerning T47D, if the CDK4/6i was added the proliferation was reduced a 27 % in T47Dr-R and a 48 % in the T47D parental cell line, being the difference in the proliferation rate significant (p-value: 0.008). However, no differences were found when both cells were cultured with fresh-DMEM (Figure 42 B).

Thirdly, MCF7p-R and T47Dr-R were cultured with the IC50 of both CDK4/6i for a week to confirm cross drug efficacy by MTT assay. The proliferation rate was reduced a 31 % when MCF7p-R was cultured with Palbociclib (p-value: 0.007), while no differences were found when cells were cultured with Ribociclib, since the proliferation rate was reduced a 10 % (Figure 43 A).

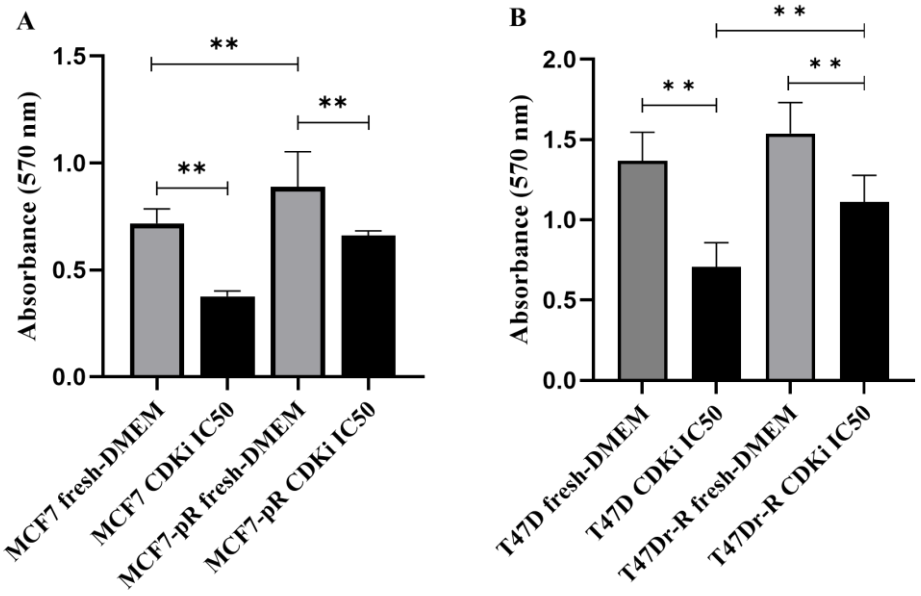


Figure 42. Proliferation assay to ensure CDK4/6i resistance acquisition. Cell were culture with CDK4/6i and fresh-DMEM for a week. A) MCF7 parental and MCF7p-R cell lines were culture with fresh-DMEM and Palbociclib IC50 (n=3 replicates) and B) T47D parental and T47Dr-R cell lines were culture with fresh-DMEM and the Ribociclib IC50 (n=3 replicates). Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

Concerning T47Dr-R, the proliferation rate was reduced a 43 % when T47Dp-R was cultured with Ribocicllib and 41 % when cultured with Palbociclib (p-value: 0.002) (Figure 43 B)

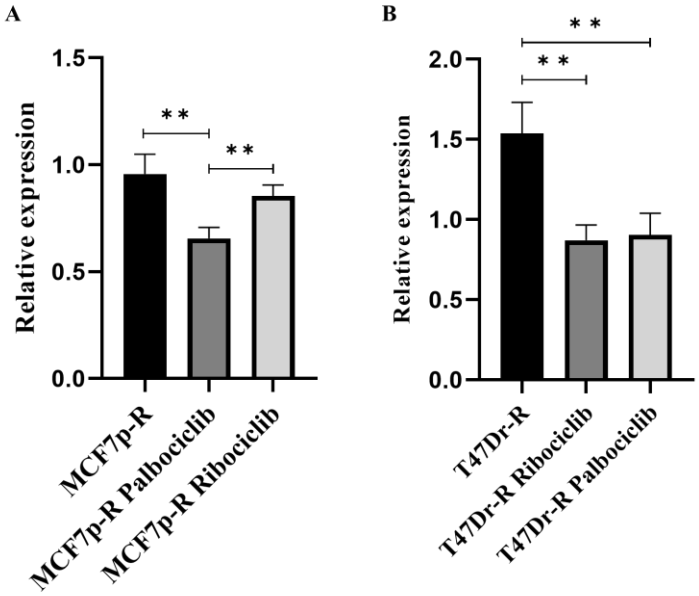


Figure 43. Proliferation assay to confirm CDK4/6i cross drug efficacy after resistant acquisition. Cells were cultured with both CDK4/6i (Palbociclib and Ribociclib) for a week A) MCF7p-R (n=6 replicates) and B) T47Dr-R (n=6 replicates). Statistics were performed by Wilcoxon test (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***)).

It was observed that both resistant cell lines after CDK4/6i resistance acquisition grew forming more colonies than the parental counterpart. To further investigate the colony formation capacity, a colony forming assay was performed to compare the MCF7-pR, T47D-rR and their corresponding parental cell lines. For that, cell lines were plated and treated with the corresponding CDK4/6i IC50 or fresh-DMEM for a week. After this period, the drug was removed and colonies were allowed to grow for an additional week for MCF7p-R and two weeks for T47Dr-R

The tumor resistant cell lines formed more colonies than the parental counterparts when culture with both fresh-DMEM and CDK4/6i IC50 (Figure 44) (Figure S11).

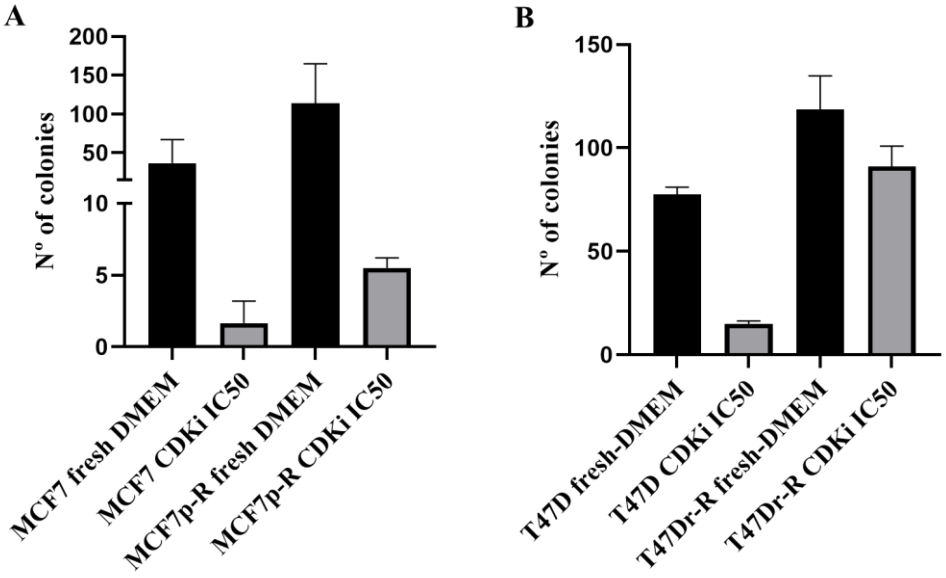


Figure 44. Colony formation assay after treating parental and resistant cell lines with CDK4/6i. A) MCF7 parental and MCF7p-R cell lines were cultured with Palbociclib IC50 for a week (n=2 replicates) and B) T47D parental and T47Dr-R cell lines were cultured with Ribociclib IC50 (n=2 replicates).

8.2. Expression of resistant -related genes in CDK4/6i resistant tumor cell line

Next, to confirm that the generated resistant cell lines were a convenient BC model for studying CDK4/6i resistance in HR+/HER2- stage IV BC, genes related to polytherapy response and resistance previously identified in CTCs (see section 2.1., Results) were studied in the resistant cell lines. As a reminder, the CTCs expression of *STAT3* and *MAPK3* before starting polytherapy was higher in patients who did not respond to polytherapy, then, in patients with intrinsic resistance. At disease progression, *BAX*, *EZH2*, *HDAC6* and *CDC7* expression was higher while *NFKBIα* and *SNAIL1* was lower in patients with acquired resistance. Moreover, genes related with the *CCND1-CDK4/6* axis were also analyzed since changes in their expression were related with drug resistance.

The figure 45 A represents the expression of the aforementioned genes in MCF7p-R cell line and its parental counterpart. The expression of *STAT3*, *MAPK3* and those genes whose expression was increased at disease progression (*BAX*, *EZH2*, *HDAC6* and *CDC7*) was higher

in the MCF7p-R cell compare with the parental cell line. On contrary, *NFKBIA* and *SNAIL1*expression was also higher in the resistant cell line. Likewise, genes related with the *CCND1-CDK4/6* axis, INK family inhibitors (*CDKN1A*, *CDKN1C*, *CDKN2A* and *CDKN2C*) or other cyclins that could overcome *CCND1* dependence (*CCND3* and *CCNE2)* were highly expressed in the tumor resistant cell line.

The figure 45 B represents the expression of aforementioned genes in T47Dr-R cell line. The expression of *STAT3* was higher in the resistant line. Nevertheless, the expression of *MAPK3* and those genes which expression was increased at progression (*BAX*, *EZH2*, *HDAC6* and *CDC7*) was higher in the parental cell line. Moreover, *SNAIL1*, *CCND1-CDK4/6* axis related genes, INK family inhibitors or other cyclins that could overcome *CCND1* dependence were also higher expressed in the parental cell line, except for *CDK6*.

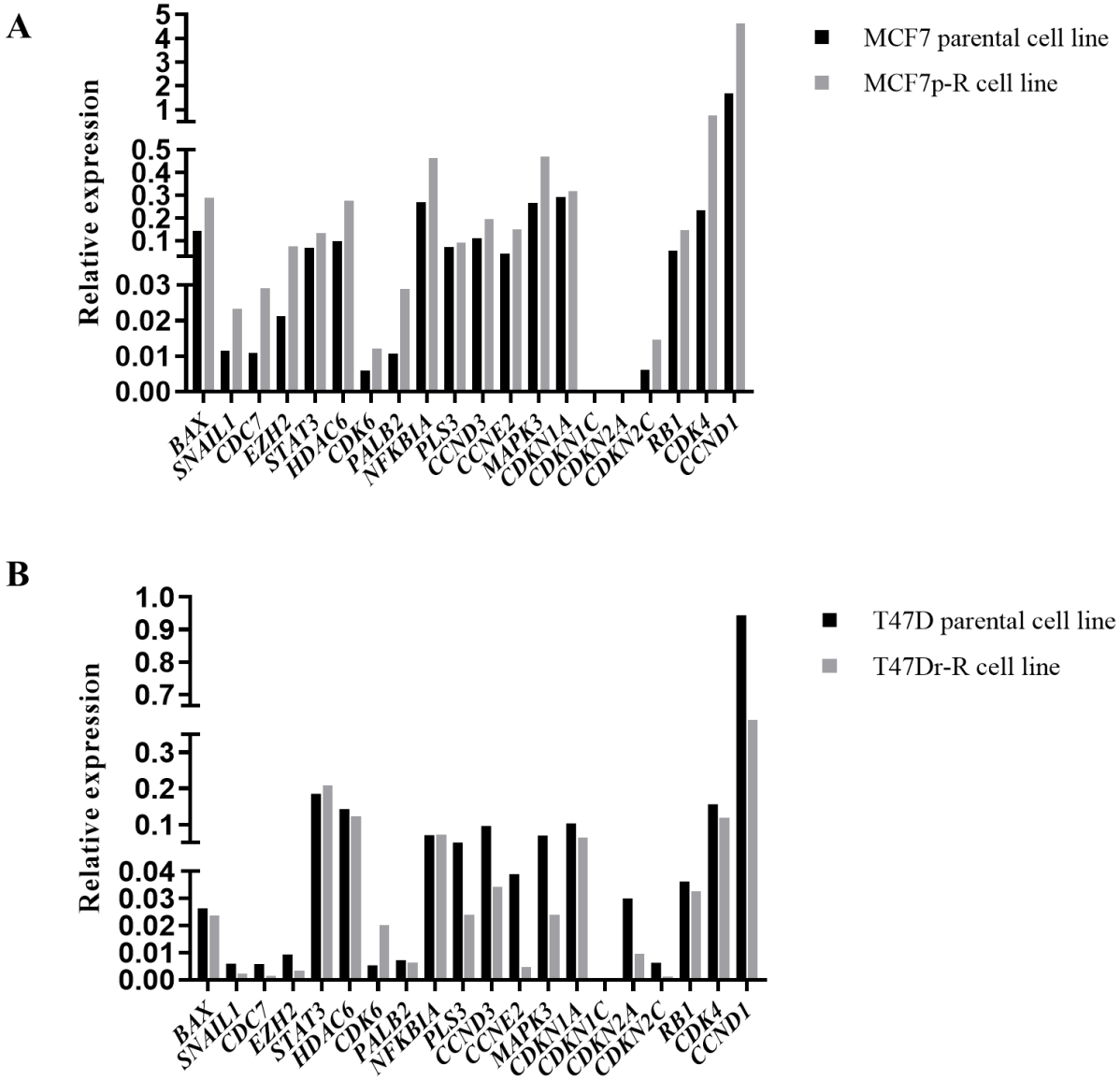


Figure 45. Gene expression of CDK4/6i resistant cell lines. A) Gene expression of MCF7p-R cell line and B) Gene expression of T47Dr-R cell line. Tumor cell line expression was relativized to *B2M*. n=2 biological replicates.

DISCUSSION

DISCUSSION

The clinical management of HR+/HER2- stage IV BC patients suffered an important advanced in 2017 due to the approval of CDK4/6i plus ET as first-line treatment. This combinatorial therapy increased the survival for more than 2 years with a few or no secondary effects. Nevertheless, 20 % of patients have intrinsic resistance within 6 months after therapy initiation, while the rest of patients will eventually acquire resistance[169]. Up to now, there is no biomarker to determine therapy response or detect therapy resistance. Moreover, the best therapy option after CDK4/6i plus ET resistance is not well-established. All things considered, the aim of this thesis is to identify biomarkers in the circulating material to tailor HR+/HER2- stage IV BC patient's therapy.

Liquid biopsy detects changes in the molecular profile of primary and/or secondary tumors in a non-invasive and real-time approach. Then, it is gaining importance in the oncology field to decipher the molecular tumor characteristics through the disease. Although the most studied tumor-entity is the ctDNA, the CTCs are promising markers, since are intact tumor cells that reflect the real tumor characteristics [1,21,85]. Up to now, CTCs enumeration is a prognostic marker in BC, but this approach does not inform about tumor molecular characteristics. For that, the analysis of CTCs transcriptome lets identify biomarkers related to prognosis, therapy response and resistance. Regarding the CTCs mutational level, it allows deciphering the tumor heterogeneity and map the clonal evolution. Then, the characterization of CTCs might be an advance in the field of precision oncology, since it would have the potential to guide and tailor patient therapy.

In this study, CTCs were isolated with a negative-enrichment method, in order to avoid the bias introduced by marker selection[40], and then genetically characterized at different time points. Besides, changes in cfDNA levels were analyzed to monitor the disease along with ctDNA mutational status. Additionally, as it has been reported that the TBME can convert immune cells into tumor promoting immune cells[96], it was evaluated the PBMC expression as an alternative marker to determine BC risk and disease management.

A total of 54 HR+/HER2- stage IV BC patients that started CDK4/6i plus ET were included in this study. Blood samples were collected when the disease was diagnosed (visit 1), after one-cycle therapy (visit 2), at disease progression (visit 3) and every 3 months after therapy initiation. In the present study, CTCs were detected by the Cellsearch® system in a 40% of patients at metastasis diagnosis or disease progression. However, the percentage of patients with CTCs detected in the peripheral blood is lower compare with what is described in the literature (50-70 %)[28]. This discrepancy should be explained for the small patient cohort analyzed (n=12 at visit 1 and 2, n=7 at visit 3). In spite of the latter, the presence of ≥ 1 CTC

after one-cycle therapy is linked with shorter survival, in agreement with Galardi *et al*.[170] and with Janni *et al* (manuscript under review), whose thresholds were ≥ 5 CTCs and ≥ 1 or 5 CTCs, respectively. Therefore, it is important to consider the number of CTCs in the peripheral blood to determine patient's prognosis. However, this approach does not decipher the CTC biology.

The main hindrances in CTC characterization are their scarcity in the circulation along with the isolation methods based on biomarkers expression, what hampered the selection of all CTC subpopulations[85]. To overcome the above-mentioned problems, the CTCs were isolated depleting the unwanted blood cells. Thus, the CTC population in our cohort shows an epithelial-mesenchymal phenotype with expression of stem cell markers at visit 1, 2 and 3, depicting the gene expression changes through the disease. It is observed that *ALDH1A1* has a higher expression at visit 3 compared with the other visits, associating with worse patient's outcome. Agreeing with this result, other studies made in CTCs from stage IV BC patients showed that CTCs with epithelial-stem features had more aggressive phenotype and associated with disease progression and therapeutic failure[171,172]. In addition, the expression of *SNAIL1* is significantly higher at visit 1, positively associating with *VIM* expression. The latter shows that our CTC population might undergo a partial EMT phenotype, what promotes cell invasion and metastasis formation in BC[173]. The expression of *CDH1* is found in 95 % of samples at different time points without greater variations, what agrees with our previous findings in CTCs from breast and prostate cancer[172,174]. It should be noticed that most of patients have a ductal carcinoma, which is characterized for the expression of *CDH1*. Hence, it was expected a concordance in CTC expression. In our results, high *CDH1* expression at visit 2 associates with polytherapy response. Similarly, Padmanaban *et al.* found that HR+ ductal carcinomas with *CDH1* expression function as a survival factor, while its lost associates with worst prognosis, metastasis formation and shorter OS in advanced BC [175,176].

To further characterize the CTCs, their transcriptome was studied using the nCounter technology to identified biomarkers related with therapy response and resistance. It is important to highlight that this approach has not been reported in CTCs from BC, although it has been reported in CTCs from hepatocarcinoma and prostate cancer [177,178]. It was chosen the PanCancer Pathway Panel over a specific Breast Cancer Panel to analyze more critical signaling pathways involve in tumor biology, tumor microenvironment and immune responses. Thus, our study determine that the nCounter assay accurately analyzed the gene expression of CTCs from BC samples without the need of a preamplification step.

After the transcriptomic analysis, the genes differentially expressed between *non-responder* and *responder* patients were analyzed by RT-qPCR in CTCs from a larger cohort. At visit 1 and 2, it was compared the expression of genes related to CDK4/6i plus ET response between *non-responder* and *responder* patients. Next, the gene expression of *responder* patients was compared between visit 1 and 3 to identify acquired resistant mechanisms. Likewise, the expression of resistant-related genes was compared between patients with intrinsic or acquired resistance at visit 3, to confirm if the resistance mechanism to CDK4/6i plus ET are common.

Before starting therapy, it is found that *STAT3, PRKCB* and *MAPK3* have a higher expression in *non-responder* patients, while *CDK6* and *CCND1* have a higher expression in *responder* patients. Likewise, *DUSP5, CDK4* and *PALB2* are prognostic markers of therapy

response. Interestingly, it is found that *STAT3*^{high}*PRKCB* ^{high}*CDK6*^{low} is a signature that classified patients according to therapy response. This signature has been tested in two additional HR+/HER2- stage IV BC cohorts. One was an internal validation cohort, then, the CTCs were also isolated with RosetteSep. The other was an external validation cohort, in which CTCs were isolated using the Adnatest instead of RosetteSep. The results demonstrate that the identified signature classified accurately HR+/HER2- stage IV BC patients according to therapy response regardless of the CTC isolation method and the sample origin. The signature can fail classifying the *initial* responder patients since, in some cases, the disease progression starts to be evident but the recurrence confirmation surpasses the 6 months threshold.

Concerning the markers that predict no response to polytherapy, *STAT3* is a powerful proto-oncogene involved in many processes for BC development[179,180]. In *in vivo* BC models, the expression of *STAT3* in the primary tumor correlated with the amount of cfDNA and CTCs released in the peripheral blood[20]. In our data, no correlation is found between *STAT3* expression from CTCs with the number of CTCs or cfDNA levels. Likewise, its expression is not detected in primary tumor samples from our cohort (data not shown). However, it associates with shorter survival at disease progression. A higher expression of *STAT3* was already reported in HR+ BC patients who progressed on Palbociclib and HR+ PDX resistant model [156]. Currently, it is being study the use of STAT3 inhibitor to revert CDK4/6i and/or ET response in HR+/HER2- stage IV BC patients who progressed on polytherapy[181–183]. Therefore, *STAT3* expression should be considered for therapy selection in HR+/HER2- stage IV BC patients when the metastatic disease is diagnosed and not only when progression occurs.

PRKCB mediates various cancer physiological processes[184–186], associating with an aggressive BC phenotype, but not with worse survival[187,188]. The latter agrees with our results, since high expression of *PRKCB* is detected in CTCs from *non-responder* BC patients[189], but it did not associate with patient's outcome. Another study made in CTCs and solid tumors found that *PRKCB* was differentially expressed in resistant BC patients, then it was included in a risk score signature to determine BC prognosis and metastatic rate[190]. Therefore, *PRKCB* expression in CTCs can be used as a marker of no response or intrinsic resistance.

Regarding *MAPK3*, its expression promotes proliferation and bone metastasis in *in vitro* and *in vivo* BC models[191,192]. In our data, no association is found between CTCs' *MAPK3* expression and bone affection, since there is a bias towards *responder* patients and bone metastasis (70 % of patients). Agreeing with our results, MAPK activation generates not only endocrine resistance but also CDK4/6i resistance[193,194]. The latter can be caused for the lack of *DUSP5* expression in *non-responder* patients, since this gene avoids MAPK pathway activation[195]. In fact, in our CTC data high expression of *DUSP5* associates with longer response to polytherapy and correlates with longer PFS. Consequently, aberrantly expression of both genes play a critical role in therapy sensitivity and cancer progression. It has been studied the combination of CDK4/6i with MAPK inhibitors to regulate transcription, as well as to block cell cycle progression[196–198], but further exploration on therapeutic interventions targeting *DUSP5* and *MAPK* are required in order to avoid CDK4/6i resistance.

Altogether, an enhance expression of *STAT3*, *PRKCB* and *MAPK3* in the advanced disease promotes CDK4/6i plus ET resistance. However, further research on CTC gene expression is needed. In addition, a deeply investigation is required to validate the inhibitors developed

against those genes as an alternative therapy for those patients who do not respond to CDK4/6i plus ET or try a triplet-combination therapy to delay resistance.

In our study, it was also found a higher expression of *CDK4* and *6* in patients with longer response[196,199], it means, patients that were treated with the polytherapy for more than 2 years. In fact, it is also found a positive correlation between the expression of both kinases at visit 1. As it is described in the literature, at visit 1, *CDK6* acts as a tumor suppressor avoiding cell-cycle progression and inducing senescence. Likewise, high *CDK4* expression is needed for a better efficacy of CDK4/6i plus ET[200]. However, in the PALOMA-3 trial both kinases were not informative of therapy response in the ctDNA of HR+ stage IV patients[70], but the expression of CTCs was not studied. Regarding *CCND1,* its expression is higher in *initial responder* patients, it means, patients who respond to polytherapy between 6 months and 2 years. *CCND1* works as an oncogene[199,201–203], thus, it might act as a tumor promoter in this group of patients. In fact, its high expression due to an increase in the copy number variations associated with poor prognosis and endocrine resistance in the ctDNA and CTCs from HR+ BC patients[204–208]

On the whole, the presence of genes involved in the CCND1-CDK4/6 axis is important for the efficacy of CDK4/6i therapy, since there are more drug targets available. Not many studies determine the expression of these genes in the ctDNA or CTCs, although our results are in line with what was observed in primary tumor or cell lines. Then, their expression can be studied to guide therapy selection.

After one-cycle therapy, it is also found relevant predictive biomarkers in CTCs. *CDKN1C* and *CUL1* have a higher expression in *non-responder* patients, while *CDH1* in *responder* patients. *CDKN1C* is a tumor suppressor gene with low levels in BC tissue, what associates with shorter OS and tumorigenesis[209–212]. After one-cycle of CDK4/6i plus ET, high levels of *CDKN1C* in CTCs associate with no polytherapy response and shorter PFS. It was described that overexpression of INK genes prevents the binding of CDK4/6i to CDK4/6, proving that the cell cycle progression is independent of CDK4/6 complex[137]. For example, in our CTC data it is observed that the cell cycle inactivation by CDK4/6i is overcome through the expression of *CDC7* in patients with acquired resistance at visit 3. *CDC7* is a kinase that controls the entry into the S phase of cell cycle[213,214]. As in our results, its high expression associates with worse prognosis and therapy resistance in Palbociclib-resistant BC tissue[215] . As its inhibition stops cell cycle in the S phase, leading to cell death without any signals to activate the cell cycle progression[216], *CDC7* inhibition is a promising anticancer agent when CDK4/6i resistance appears.

CUL1 is a key component of the SCF ubiquitin ligase complex, responsible for the degradation of various cellular proteins. In BC tissue, *CUL1* overexpression correlates with cancer progression and worse OS[217]. It is described that *CUL1* controls tumor growth and metastasis formation through the PI3K/AKT/mTOR pathway[218,219]. In fact, in our data it is found a positive association between *CUL1* and *PI3KCG* expression at visit 1, which could indicate that cancer cells continue growing. No data was found in the literature related to its role on therapy response.

More studies on *CDKN1C* and *CUL1* expression in the tumor circulome are needed, but their high expression in patients who do not respond to CDK4/6i plus ET at visit 2 could indicate that alternative pathways are controlling the cell cycle and promoting metastasis.

After one-cycle therapy, high levels of *PALB2* in CTCs associate with longer OS. Contrary, its higher expression in tumor tissue as well in CTCs from stage IV BC patients correlated with worse outcome[172,220]. The above-studies did not consider CDK4/6i therapy, then it could be suggested that the role of *PALB2* to determine BC prognosis is dependent on the therapy regimen. Moreover, it is well-described the role of *PALB2* as a G2/M phase regulator[221–223]. Hence, its overexpression could show the efficacy of CDK4/6i retaining the cell cycle in the G1/S phase, enhancing the survival of HR+/HER2- stage IV BC patients.

The CTC gene expression analysis also allows the identification of resistant-related genes involved in cell proliferation and cancer development such as *BAX*, *EZH2*, *HDAC6*, *PLAU*, *RELA*, *CDC7*, *NFKBIA* and *SNAIL1*. Moreover, it was possible to identify genes specifically implied in intrinsic (*CASP8*) and acquired resistance (*HDAC6*, and *CDC7*). The expression of these genes was checked in two CDK4/6i resistant cell lines: MCF7p-R and T47Dr-R, as an *in vitro* BC model. The gene expression of the MCF7p-R represents the CTC expression from our cohort at disease progression more accurately than T47Dr-R. For example, it was observed a higher expression of epigenetic (*EZH2* and *HDAC6*), INK family inhibitors (*CDKN1A* and *CDKN2C)* and cell-cycle related genes (*CDK6*). Likewise, the expression of genes related to no-response to CDK4/6i plus ET (*STAT3* and *MAPK3*) in our cohort is also enhanced in the MCF7p-R cell line. Additionally, as it is described in the literature, genes that control the cell cycle after CDK4/6i resistance acquisition (*CDC7*, *CCNE2* and *CCND3*) have a higher expression in the BC model. The MC7p-R cell line appears to be more similar to our cohort on one hand, due to the ER+ hormonal status, while T47D is a progesterone-dependent cell line. On the other hand, there is a bias towards Palbociclib therapy in our cohort. So, it could be possible that the transcriptomic changes depend on the CDK4/6i used. Additionally, although both cell lines are considered as slow-growing cells, MCF7 doubling time is minor than T47D. The latter could reduce or retard drug efficiency in stopping the cell cycle in the T47D, and consequently, the acquisition of the resistance phenotype. Therefore, the generation of resistant cell line protocol might be adjusted to cell characteristics. In addition, the MCF7p-R cell line can be used in the future to test therapy options for HR+/HER2- stage IV BC patients after resistance acquisition

Both *BAX* and *CASP8* are well-known for its apoptotic function[224,225], although these genes also have an anti-apoptotic role promoting BC progression[226–229], increasing cell motility, metastasis formation and therapy resistance[230–232]. We find a positive association between *BAX* and *CASP8* expression at disease progression. Likewise, both genes seem to have a role in therapy resistance, being *CASP8* highly expressed in patients with intrinsic resistance, it means, patients who do not respond to CDK4/6i. Additionally, these genes are highly expressed in patients with wild-type *PI3K*. Hence, a deeply understanding of their non-apoptotic function in advanced BC could help to identify altered signaling pathways that restore the cell cycle progression in spite of CDK4/6i administration.

Interestingly, two epigenetic regulators have an enhance expression at disease progression, *EZH2* and *HDAC6*. *EZH2* is a histone methyltransferase which high expression associates with

poor outcome in stage IV BC[233–235]. Its expression is regulated by *CUL1* through the PI3K/AKT/mTOR pathway. In our results, *EZH2* is highly expressed in patients with acquired resistance to CDK4/6i plus ET, agreeing with Abu-Khalaf *et al.* results in the ctDNA from stage IV BC patients resistant to Palbociclib[236]. Apart from high *EZH2* expression, we observe a high expression of *CUL1* in patients with *PI3K* wild-type. As *CUL1* correlates with no therapy response at visit 2, it is likely that this pathway might be driven CDK4/6i plus ET resistance in HR+/HER2- stage IV BC patients. However, further research is required to fully elucidate the underlying molecular interactions among these genes. Concerning *HDAC6*, it is a histone deacetylase regulated by estrogen signaling that promotes BC proliferation, differentiation and survival[237]. At disease progression, *HDAC6* expression was specifically increased in patients with acquired resistance, and mainly when *PI3K* was mutated. The latter is likely to have received the polytherapy for more than 6 months. Currently, it is being studied the efficacy of combining HDAC6 inhibitors with PI3K inhibitors and with Palbociclib to explore novel treatment avenues for HR+/HER2- BC patients[234,238,239].

Considering the above-results, in addition of the CTCs transcriptome it should also be studied the epigenetic silencing of metastatic suppressor genes, since it is important for CTCs survival, metastatic formation and therapy efficacy[240,241]. Then, epigenetic markers in the tumor-derived material require further study as a liquid biomarker for stage IV BC patients follow-up, as it was previously reported for our group in the cfDNA from HR+ stage IV BC patients[242].

Agreeing with our results, *CDK6* overexpression or amplification occurs in CDK4/6i resistance models, such as in Abemaciclib resistant ER+ BC cell line[243] and in the ctDNA from ER+ BC patients that underwent disease progression on Palbociclib plus ET[144]. On the contrary to what is observed at visit 1, at radiological progression, *CDK6* acts as a tumor promoter through its non-catalytic activity. For example, phosphorylating *RELA* to activate the transcription of genes related to inflammatory response and EMT[244,245]. In our case, it is observed not only a higher expression of *CDK6* and *RELA*, but also a reduced expression of its inhibitor, *NFKBIα,* in patients with long therapy response and hence, acquired resistance. In fact, it was studied as a therapeutic approach to avoid the binding of *RELA* with other *NF-kB* factors to restore CDK4/6i response. All things considered, in our cohort the accumulation of *CDK6* has a dual role depending on the time point studied, being extremely important in those patients treated with the polytherapy for more than 6 months. Likewise, its role controlling *NF-kB* factors required a deeper understanding, since this pathway could be interesting for restoring therapy response to CDK4/6i plus ET.

PLAU, the urokinase plasminogen activator, is involved in tumorigenesis and metastasis in BC[246,247]. It is a poor prognostic marker found in CTCs from stage IV BC patients showing disease progression[248]. It was also found upregulated in CTC-clusters from an *in vivo* BC model as a mechanism to improve CTC survival in the circulation[249]. Thus, CTC expression of these genes could represent those CTCs able to survive in the circulation. No data was found related to CDK4/6i. Hence, a deeper understanding of this gene in therapy resistance is required.

In our CTC data, the expression of *SNAIL1* changes through disease. Firstly, its expression positively associated with *VIM* expression at visit 1, indicating a partial EMT phenotype, promoting cell invasion and metastasis formation in BC[173,250,251]. The EMT program is boosted

when *JAK2* activates the PI3K/AKT/mTOR pathway which in turn, activates mesenchymal genes as *SNAIL1* and *VIM*[252]. The latter agrees with other results observe at visit 1, a positive association between *JAK2* with *PI3KCG* and *VIM*. Likewise, the association is almost significant between *SNAIL1* with two subunits of *PI3K* (*PI3KCG* and *PI3KCA*). After one-cycle therapy, *SNAIL1* expression significantly decreased, which agrees with one study made in a BC xenograft model, where Palbociclib treatment decreased *SNAIL1* at protein level[253]. Lastly, in *long* responder patients *SNAIL1* expression is significantly lower at visit 3 compare with visit 1. Therefore, the low expression of this gene after CDK4/6i administration could indicates a longer response to CDK4/6i plus ET, probably because cancer cells have a less aggressive phenotype and consequently, less invasion capacity.

The above-mentioned results prove the temporary heterogeneity of the CTC population in our cohort and how the tumour molecular characteristics evolve during disease. As far as we know, this transcriptomic analysis was the first one reported in CTCs from HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET at different time points. It is confirmed the importance of CTCs for identifying biomarkers related to polytherapy response and potential resistance mechanisms, although we could not differentiate ET and CDK4/6i resistance mechanisms. However, our group demonstrate that is feasible to characterize CTCs to identify potential future alternative therapy lines or new targeted therapies to beneficte this patient group. In line with this, an accepted subsequent therapy after CDK4/6i resistance is Alpelisib, that accurately reduces the mutant *PI3K* copies[154,254]. In our cohort the majority of patients with mutant *PI3K* at disease progression were *responders*, on the contrary to the literature, in which *PI3K* alterations increase the risk of having CDK4/6i resistance in a few months[255,256]. As in MONALEESA-7 trial, in our study no significant association between the *PI3K* mutational status and survival is detected, although it does in PALOMA-3 and MONALEESA-2 trial [128,139,257]. However, a bigger cohort is needed to deeply confirm this result, since in our cohort there is a bias towards *responder* patients. Although it would have been interesting to study the subsequent benefit from receiving Alpelisib after CDK4/6i resistance, just a few patients were treated with the PI3K inhibitor. Additionally, in our cohort the CTC gene expression associated with *PI3K* mutations in the ctDNA from HR+/HER2- stage IV BC patients.

Considering the clinical relevance of different mutations, a discovery mutational analysis was done sequencing the CTCs' DNA from HR+/HER2- stage IV BC patients to determine predictive alterations of polytherapy response. Thus, our study determines that is possible to sequence the CTCs' DNA from BC samples. Nevertheless, a larger cohort in the discovery analysis and a deeper informatic analysis would be required to precisely select and define the information obtained. In spite of the latter, interesting alterations are observed. For example, it is found that all patients shared the same mutation in *KMT2C* and *C/EBPα*. *KMT2C* is a histone methyltransferase protein involved in the regulation of gene transcription[258]. Pathogenic variants in *KMT2C* are commonly found in HR+ stage IV BC patients[259–261], correlating with disease recurrence[258,262] and shorter PFS on ET, supporting its role in endocrine resistance[260]. In our cohort, it is found a pathogenic stop gaining mutation before therapy initiation. Considering the literature, pathogenic alterations in *KMT2C* increase the sensitivity towards PI3K inhibitor plus PARPi treatment [263] and EZH2 inhibitors[264]. Hence, its detection could be considered to treat patients with Alpelisib or EZH2 inhibitors along with CDK4/6i.

It is common that HR+ BC patients had a loss of function in *ARID1A* gene, which regulates cell cycle and/or mediates endocrine resistance along with *C/EBPα*[265–272]. Mutations in both *ARID1A* and *C/EBPα* are detected in *non-responder* patients, what could explain the prompted disease progression and no response to therapy in our cohort. In fact, it has been proposed the use of CDK4/6, PI3K, EZH2 or HDAC6 inhibitors to treat mutated-*ARID1A* stage IV BC[,266,273,274], which reinforces the pathways altered previously identified in our CTC data. An in-frame deletion in *ARID1B* was identified in the *responder* group. In spite of belonging to the same family, *ARID1A* and *ARID1B* have opposite roles in cell cycle regulation. *ARID1B* promotes cell cycle progression[275,276], but it is inactivated in BC recurrence what in turn suppresses the proliferation of *ARID1A*-deficient in BC[277]. Then, alterations in *ARID1A* and *C/EBPα* could be markers of no benefit from CDK4/6i, while *ARID1B* mutation predicts better prognosis and longer response in HR+/HER2- stage IV BC patients treated with CDK4/6i plus ET.

It is also identified an *HSP90AA1* in-frame deletion in CTCs from *non-responder* patients. In the literature, *HSP90AA1* alterations correlate with risk of BC in early stages and metastasis in advanced disease in HR+ patients [278,279], as well as an increased in the CTCs' metastatic potential in prostate and ovarian cancer[280]. However, we detect the alteration in the ctDNA from both *non responder* and *responder* patients, hence, it does not have any biological implication in therapy efficacy in our cohort. On one hand, the small cohort analyzed could generate a bias in the results, since it does not represent the whole population. On the other hand, the variant allele frequency was low and no amplification step was performed, then, it is likely that some alterations in the CTC samples were not detected. Altogether, an informatics analysis, literature investigation as well as a bigger cohort are needed to understand the implications of the mutations on treatment response. On the whole, it was confirmed that CTCs can be used to identify tumor predictive mutations. Besides, it is shown that CTCs and ctDNA bear concordance information concerning the genetic tumor profile. Then, the ctDNA can be analyzed to monitor mutations found in CTCs. In addition, this study proves that combining transcriptional and mutational information from CTCs gives a widely vision of pathways altered in HR+/HER2- stage IV BC patients.

Although the CTC represents the tumor biology and has potential clinical implications, this entity has been little explored compared to the ctDNA. Both cfDNA and ctDNA have various applications in cancer management, such as longitudinal monitorization of clonal evolution, detection of minimal residual disease, determination of drug response and disease progression, etc[281]. The longitudinal study of cfDNA every 3 months is not enough to get ahead of disease progression. However, as it was previously reported by Fuentes-Antrás *et al*[254]., low cfDNA levels associate with better prognosis at 3 and 6 months. Moreover, patients are accurately classified according to therapy response considering both CDR and cfDNA concentration at 6 months. It remains uncertain whether the monitorization of cfDNA has a clinical significance or can complement the radiological data. In this regard, prognostic results were obtained earlier (at day 15 or 30 after therapy initiation) studying the number of mutant copies in the ctDNA[70,71]. Therefore, a change in the sampling strategy should be considered, increasing the number of patients, the transient shifts collected and tracking the ctDNA mutational dynamics instead of cfDNA levels. No association is found between cfDNA or ctDNA and patient's clinical variables.

To complete the study, PBMCs were analyzed, since TBME could transforms the immune cells into malignant immune cells, leading to immunosuppressive conditions[2]. It has been described that specific leukocyte levels correlates with clinical parameters such as subtype, number of CTCs, metastatic burden and disease progression[282,283]. In our case, the leukocyte levels in HR+/HER2- stage IV BC patients decreased after one-cycle therapy. In addition, the type of leukocyte altered differ among patient groups. Hence, it is suggested that each leukocyte could have a specific role in polytherapy response. In line with the latter, the NLR is studied, finding that after one-cycle therapy the immune fraction levels were recovery in the HR+ treated with CDK4/6i plus ET. A deeply study is needed, but NLR could be used to determine the immune response of BC patients. No association between clinical parameters and leukocyte levels is found. However, it is proved that the transcriptome of PBMCs can be studied to determine BC prognosis and therapy efficacy. Thus, it is observed that *GZMB*, *KLF4* and *MYCL* have a higher expression in *responders* than *non-responder* patients before therapy initiation, predicting CDK4/6i plus ET response. Besides, the expression of *KITLG*, *KMT2D* and *KLF4* is lower in BC patients (stage I-IV) compare with cancer-free woman, correlating with cancer aggressiveness and suggesting and additional role as a prognostic marker. Interestingly, at disease progression the genetic alterations in the immune fraction do not differ among patient groups in the HR+ stage IV cohort.

KITLG has a role in hematopoiesis, T-cell proliferation and mast cell development. In the literature, high levels of *KITLG* in mast cells[284] and in plasma from HR+BC patients[285] associate with better outcome, probably because *KITLG* controls the immune cell survival and growth. In our PBMC data, a progressive loss of *KITLG* expression is observed among BC stages, as it was previously in malignant transformation of mammary tissue [286–289]. Hence, PBMC gene expression mimics what occurs in the mammary tissue. As the PBMC transcriptome was analyzed before therapy initiation, we ruled out the possible influence of the treatment decreasing its expression. After the paracrine co-culture with HR+ cancer cells, *KITLG* expression significantly decreases in PBMCs from cancer-free women and early-BC stages, but not in PBMCs from advanced disease. It is likely that *KITLG* expression in the immune cells is already altered due to the previous contact with TBME. Therefore, its inhibition could reduce the immune response. Secondly, studies that investigate the role of *KMT2D* in PBMCs were not found. However, its alterations affect the immune response as controls the immune checkpoint blockade in multiple cancers[290,291]. The latter affects the transcription of genes related with T-reg cell differentiation or recruitment, which within the tumor microenvironment suppress the immunosurveillance to promote tumor progression[292,293]. Lastly, *KLF4* correlates with immune system activation, inflammation, apoptosis as well as disease severity if its promoting region is silenced[294–297]. The latter agrees with our results, in which *KLF4* has a reduced expression in stage IV BC compared with cancer-free women. Likewise, in BC malignant tissue *KLF4* was found to be lower than in health tissue[298,299]. Again, PBMC transcriptome reflects the genetic tumor alterations. Nevertheless, it was observed that *KLF4* is highly expressed in macrophage type 2 polarization in the tumor microenvironment, a phenotype related with tumor cell growth, invasion, metastasis and immune activity inhibition[296,300–302]. In this study it is considered the expression of all leukocyte types, while other studies considered the expression of specific immune cells. Thus, a deeply genetic characterization of each leukocyte type could elucidate the malignant role of each immune cell

in BC development[303,304]. In the paracrine co-culture assay, a decreased in *KLF4* expression is only significant when naïve PBMCs from cancer-free women are co-cultured with HR+ cell line, pointing out that tumor cells might have an inhibitory effect on *KLF4* expression. No effect is observed in BC patient, possibly due to the previous contact of immune cells with the TBME. Additionally, *KLF4* predicts therapy response in HR+/HER2- stage IV BC patients, since a higher expression was found in *responder* patients.

Apart from *KLF4*, in our PBMC data *MYC-L* and *GZMB* also predicts patients' response to polytherapy, as its expression is highly reduced in *non-responder* patients. Agreeing with our findings, low *MYC* expression at transcriptional levels was observed in NK cells and T lymphocytes in patients with cancer, which leads to a metabolic cell arrest in G0/G1 phase and reduced immune response to antigen presentation[305–308]. On the contrary, macrophages require high expression of *MYC* to suppress the innate immunity and facilitates tumor immune escape[309,310]. As it was said before, the transcriptome of each leukocyte type should be characterized to further comprehend the action of immune cell in cancer development.

Regarding *GZMB,* its high expression correlates with good prognosis and therapy response, determining which patients will benefit from CDK4/6i plus ET. The latter agrees with what is published in primary BC tissues, in which *GZMB* is a metastatic suppressor to regional lymph nodes, which increases patient's OS [311,312]. In fact, *GZMB* expression is been used to predict therapy response in various cancer types[166,313–315]. In agreement, in our data *GZMB* expression is informative of good outcome both in the CTC-enriched fraction and in PBMCs freshly-isolated from peripheral blood. As the RosetteSep does not efficiently deplete a lymphocyte subpopulation, the *GZMB* expression detected in the CTC fraction could come from a non-depleted PBMC population. Further analysis of the lymphocyte subpopulation should be performed to determine its clinical implication. In spite of the latter, it can be said that *GZMB* expression from both entities could be considered to determine polytherapy response.

To sum up, this study is the first one to demonstrate the potential use of PBMCs as circulating marker for predicting CDK4/6i plus ET response. Nevertheless, the PBMC expression was not study with a panel that represents the immune related pathways, since PBMCs were analyzed along with CTCs to discard their background in the CTC analysis. Hence, a specific immune panel should be used to deeply characterize the PBMC transcriptome and to fully comprehend the crosstalk between the microenvironment and the tumor cells, increasing the value of our results.

This study highlights the need of using liquid biopsy as a complementary approach to tissue biopsy, and provides insights into the applicability of CTC analysis. The longitudinal CTC characterization provides precise information of altered pathways in HR+/HER2- stage IV BC patients to guide therapy selection both before starting therapy and at disease progression. In spite of the latter, additional studies (preclinical and clinical) are necessary to validate alternative therapies lines to CDK4/6i plus ET. After the CTC study, our proposal to manage HR+/HER2- stage IV BC patients would be, before therapy initiation, those patients with high expression of genes involved in the axis CCND1-CDK4/6 should be treated with CDK4/6i plus ET. However, if the CTC analysis reveals high expression of *STAT3* or *MAPK3* or low levels of *DUSP5,* STAT3 or MAPK3 inhibitors could be administered. At disease progression, PI3K inhibitors can be administered when *PI3K* is mutated as monotherapy or in combination with

EZH2 or HDAC6 inhibitors, if these epigenetic pathways are enhanced, or CDC7 inhibitors since this cyclin might regulate the cell cycle progression after CDK4/6i resistance acquisition. Likewise, patients with mutant *ARID1A* could be treated with EZ*H2* or *HDAC6* inhibitors. In addition, BC models resistant to CDK4/6i are necessary to determine the CDK4/6i resistance mechanisms, as well as to test future therapy lines after resistance acquisition. We also recommend a longitudinal monitorization of cfDNA or ctDNA to determine polytherapy response as well as therapy efficacy when a targeted-mutation is tracked, such as *PI3K* or *ER1*[254]. Lastly, we highlight the importance of further research on the circulating immune fraction in order to elucidate its prognostic and predictive function, and comprehend the effect of its interaction with tumor cells.

This study has some limitations. First, the small cohort analyzed. Additionally, there are a bias towards *responder* patients and Palbociclib treatment, which hinders the significant statistically analysis. A prolonged follow-up is needed to explore illustrative clinical groups. Second, the discovery analyses were performed in a small cohort, which does not represent the population heterogeneity and reduces the detection of informative marker. Third, the CTC enumeration data was not available in all patients and the cfDNA follow-up was not equal in the whole cohort regarding the analyzed time points, reducing the result's value. Besides, tissue samples were not available for all patients. Fourth, the PBMC expression profile was studied in a non-immune panel, reducing the identification of immune-related markers.

Challenges for future research include standardized protocols to isolate all CTCs subpopulations and reduce non-specific tumor cells enrichment, implementation of CTCs monitorization during systemic therapy, validate markers of therapy response and resistance and combined the information obtained from other circulating material. The present study provides valuable information to face the above-mentioned challenges, since informative markers were identified in CTCs. In addition, it was proved that CTCs and ctDNA bear complementary information, as well as the potential application of PBMCs in the oncology field.

CONCLUSSIONS

CONCLUSSIONS

1. The CTC characterization at transcriptional level allows the identification of response biomarkers to tailor-patients therapy. Higher expression of genes implied in the CCND1-CDK4/6 axis indicates benefit from CDK4/6i plus ET, while higher expression of *STAT3, PRKCB* and *MAPK3* as well as low *DUSP5* expression indicates no response.

2. The gene expression signature $STAT3^{high}PRKCB^{high}CDK6^{low}$ on CTCs before starting therapy classifies HR+/HER2- stage IV BC patients according to polytherapy response regardless of the CTC isolation method or sample provenance.

3. The CTC characterization at transcriptional level allows the identification of resistance biomarkers, which could guide the selection of the subsequent therapy lines, such as inhibitors against the epigenetic regulators *EZH2* and *HDAC6* and against the cell cycle regulator *CDC7*.

4. The *in vitro* model generated mimics the alterations observed in our HR+/HER2- stage IV BC cohort, which will let test new potential therapeutic targets after CDK4/6i resistance acquisition.

5. A great number of mutations are detected in the CTCs from our cohort, which impaired the identification of informative alterations regarding therapy response and resistance acquisition. Hence, a larger cohort is needed to accurately represent the population heterogeneity.

6. A higher cell-free DNA ratio (CDR) associated with patients' survival both at 3 and 6 months after therapy initiation. Likewise, the CDR and ctDNA allows the classification of HR+/HER2- stage IV BC patients according to therapy response at 6 months after therapy initiation. Then, cfDNA and ctDNA can be used to monitor therapy response.

7. Both ctDNA and CTCs bear concordance and complementary information. In this study it was observed an association between the mutational status of *PI3K* in the ctDNA with the CTC expression at disease progression. Therefore, the data obtained from both tumor-derived material should be considered together for HR+/HER2- stage IV BC management.

8. The gene expression of PBMCs allows the identification of informative biomarkers in BC. Higher expression of *KLF4*, *MYCL* and *GZMB* associates with CDK4/6i plus ET response, while lower *KITLG* expression with advanced disease.

9. The PBMC transcriptome reflects the crosstalk of immune cells with the tumor microenvironment, since the gene expression differs between naïve PBMCs and those from cancer patients, which have been previously exposed to cancer-related molecules.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

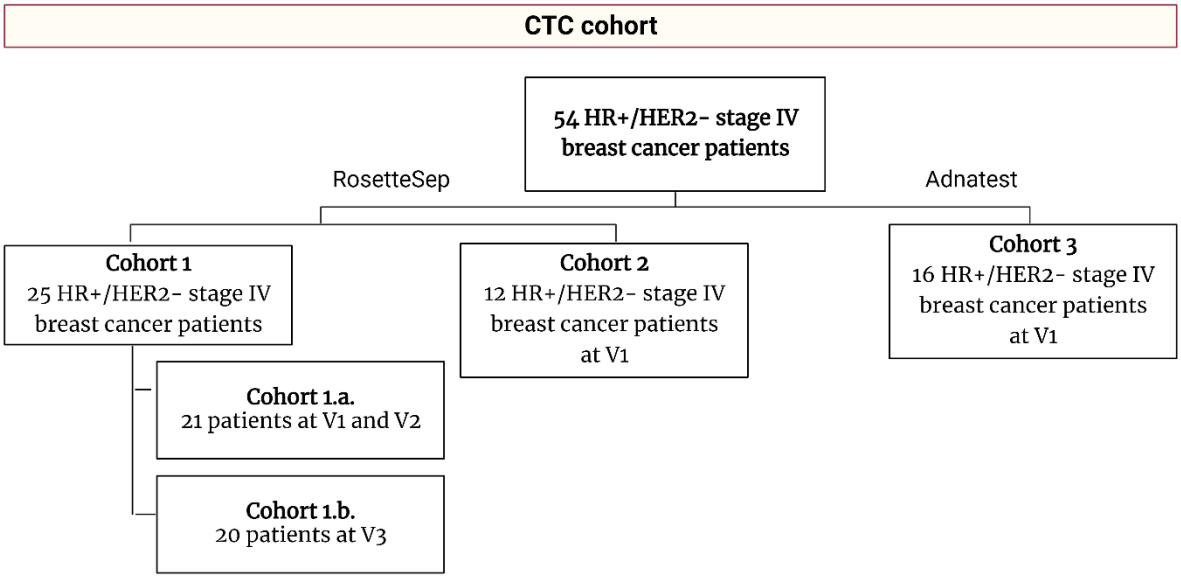


Figure S1. Scheme of HR+/HER2- stage IV BC patient's cohort in which the CTCs were studied for the identification of response and resistance biomarkers. A total of 54 patients were included. CTCs from 38 patients were isolated with RosetteSep. The cohort 1 included 25 HR+/HER2- stage IV BC patients. This cohort was divided into cohort 1.a., that included 21 patients studied at visit 1 and visit 2, and cohort 1.b., that included 20 patients studied at visit 3. The cohort 2 is an internal validation cohort formed by 13 HR+/HER2-stage IV BC patients. The cohort 3 is an external validation cohort that includes 16 patients whose CTCs were isolated with Adnatest.

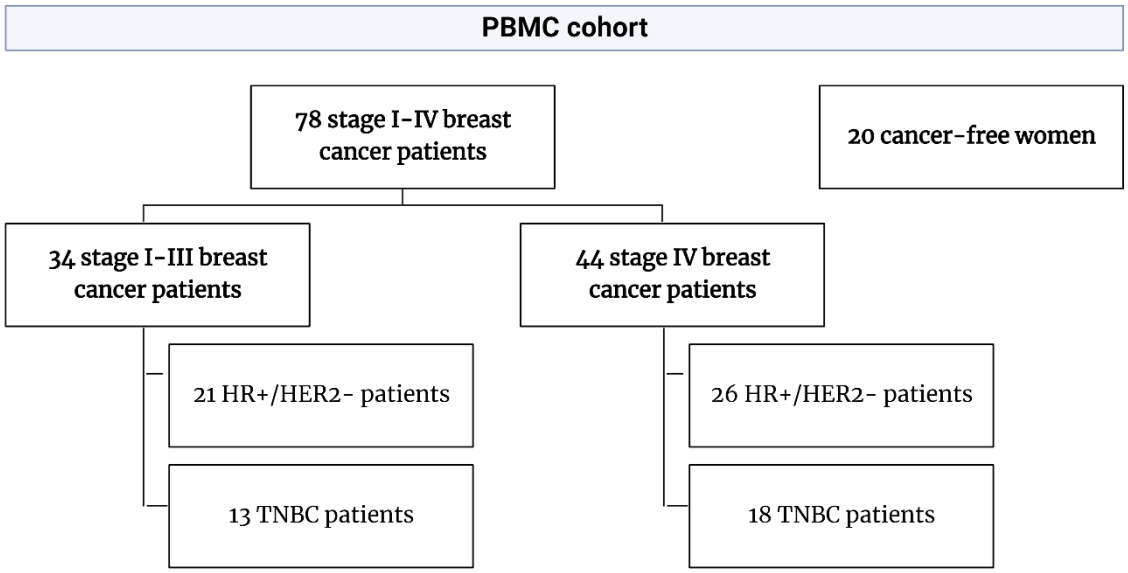


Figure S2. Scheme of patient's cohort in which the PBMCs were studied for the identification of response biomarkers. A total of 78 BC patients and 20 cancer-free women were included. PBMCs from 34 stage I-III and 44 stage IV BC patients were isolated; 21 and 26 patients with HR+/HER2- subtype and 13 and 18 TN BC subtype, respectively.

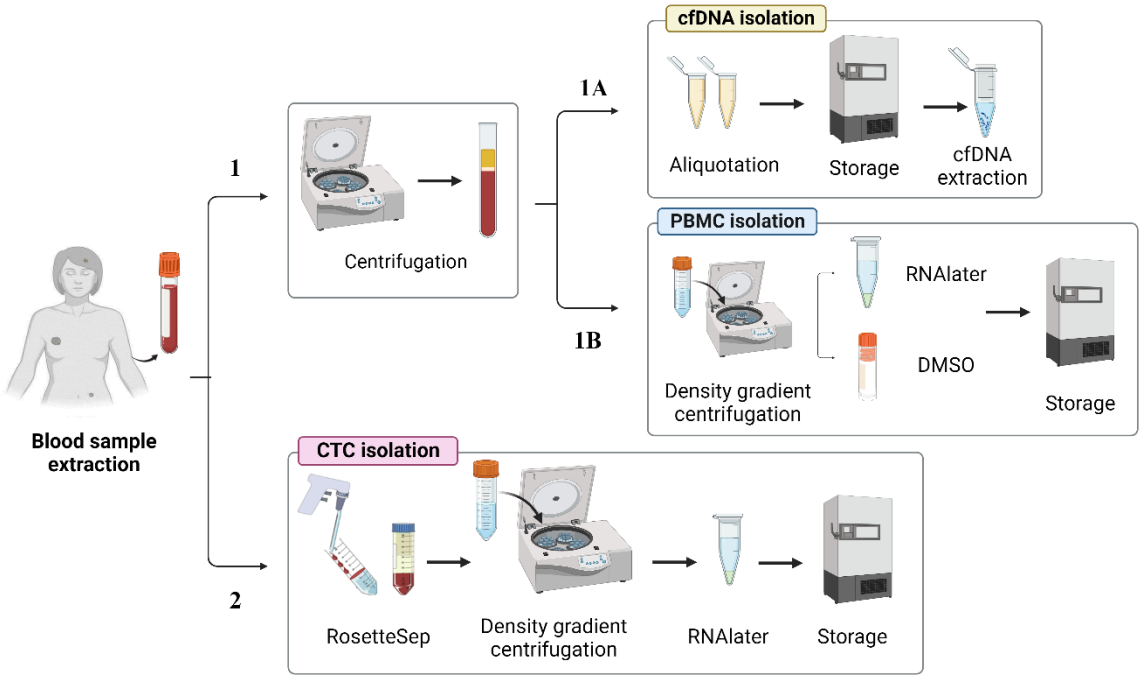


Figure S3. Workflow of tumor-derived material extraction. 1) An EDTA tube of 7.5 mL was centrifuged for phase separation. 1A) Plasma is aliquoted for cfDNA isolation using the Qiamp nucleic acid kit extraction. 1B) The white blood fraction is isolated by density gradient centrifugation using Lymphoprep. PBMCs were kept in RNA Later or DMSO for further analysis. 2) CTCs were isolated using the RosseteSep cocktail. Unwanted cell fraction was precipitated by a density gradient centrifugation. CTC enriched fraction was kept in RNA Later for further analysis.

Table S1. Genes analyzed by RT-qPCR: biological function, experimental context and circulating material analyzed

Gene	TaqMan Probe	Biological function	Experiment	Circulating material
ALDH1A1	Hs00946916_m1	Stemness	Phenotypic characterization and prognosis	CTC, PBMC
B2M	Hs00187842_m1	Reference	CTC, PBMC and cell line normalization	CTC, PBMC
BAX	Hs00180269_m1	Apoptosis	Resistance mechanisms	CTC, PBMC
CASP8	Hs01018151_m1	Apoptosis	Resistance mechanisms	CTC, PBMC
CCND1	Hs00765553_m1	Cell cycle regulation	Response mechanisms	CTC, PBMC
CCND3	Hs01017690_g1	Cell cycle regulation	Resistance mechanisms	Cell line
CCNE2	Hs00180319_m1	Cell cycle regulation	Resistance mechanisms	Cell line
CDC7	Hs00177487_m1	Cell cycle regulation	Resistance mechanisms	CTC, PBMC
CDH1	Hs00170423	Cell adhesion	Response mechanisms	CTC, PBMC
CDK4	Hs01565683_g1	Cell cycle regulation	Response mechanisms and prognosis	CTC, PBMC
CDK6	Hs04992784_s1	Cell cycle regulation	Response and resistance mechanisms	CTC, PBMC
CDKN1A	Hs00355782	Cell cycle regulation	Resistance mechanisms	Cell line
CDKN1C	Hs00175938_m1	Cell cycle regulation	Response mechanisms and prognosis	CTC, PBMC
CDKN2C	Hs00181211_m1	Cell cycle regulation	Resistance mechanisms	Cell line
CUL1	Hs01116994_g1	Cell cycle regulation and signalling	Response mechanisms	CTC, PBMC
DUSP5	Hs00244839_m1	Proliferation and differentiation	Response mechanisms and prognosis	CTC, PBMC
EZH2	Hs01016789	Epigenetic regulation	Resistance mechanisms	CTC, PBMC
GZMB	Hs00188051_m1	Apoptosis	Response mechanisms and prognosis	CTC, PBMC
HDAC4	Hs01041648_m1	Epigenetic regulation	Resistance mechanisms	CTC, PBMC
HDAC6	Hs00997427_m1	Epigenetic regulation	Resistance mechanisms	CTC, PBMC
JAK2	Hs01078136_m1	Transcriptional regulation	Response mechanisms	CTC, PBMC
KITLG	Hs00241497_m1	Hematopoiesis and immune cell development	Response mechanisms	PBMC
KLF4	Hs00358836_m1	Homeostasis and immune response	Response mechanisms	PBMC
KMT2D	Hs00912419_m1	Epigenetic regulation	Response mechanisms	PBMC
MYC-L	Hs00420495_m1	Transcription factor	Response mechanisms	PBMC
NOTCH1	Hs01062014_m	Transcriptional regulation	Resistance mechanisms	CTC, PBMC
PALB2	Hs00226617_m1	Tumor suppression	Phenotypic characterization and prognosis	CTC, PBMC
PI3KCA	Hs00907957	Oncogene	Response mechanisms	CTC, PBMC
PI3KCG	Hs00932390_m1	Oncogene	Response mechanisms	CTC, PBMC
PLAU	Hs01547054_m1	Migration and invasion	Resistance mechanisms	CTC, PBMC
PLS3	Hs00543971_m1	EMT	Phenotypic characterization	CTC, PBMC
PROM1	Hs01009257_m1	Stemness	Phenotypic characterization	CTC, PBMC

PTPRC	Hs04189704_m1	Blood cell marker	Cohort 3 normalization	PBMC
RAC2	Hs05026727_mH	Celular response regulator	Resistance mechanisms	CTC, PBMC
RB1	HS01078066_m1	Cell cycle regulation	Response mechanisms	CTC, PBMC
RELA	Hs01042014_m1	Transcriptional regulation	Resistance mechanisms	CTC, PBMC
SNAIL1	Hs00195591_m1	EMT	Phenotypic characterization	CTC, PBMC
STAT3	Hs00374280_m1	Transcription factor	Resistance mechanisms and prognosis	CTC, PBMC
TGFBR2	Hs00234253_m1	Reference	PBMC normalization	PBMC
VIM	hs00958116_m1	EMT	Phenotypic characterization	CTC, PBMC

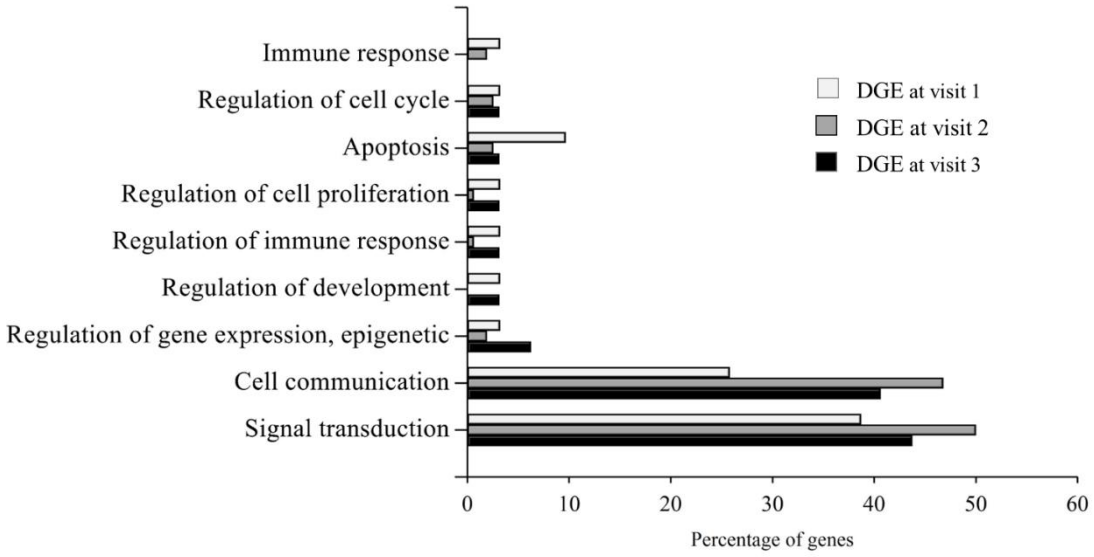


Figure S4. Biological pathways altered in HR+/HER2- stage IV BC patients at visit 1 (n=21), visit 2 (n=21) and visit 3 (n=20). A GO analysis was performed considering the differential expressed genes identified in the DESEq2 analysis from nCounter data, using the *long* responder group as a reference. (DGE: differential gene expression).

Section S1. PBMC background in the CTC-enriched fraction

The RosetteSep method for CTC isolation has an important non-specificity, then, the PBMC background in the CTC-enriched fraction affects the CTC expression detection. To further analyse which PBMC population is not depleted with RosetteSep, different PBMC isolation methods were used (red blood cell lysis buffer, Lymphoprep and RosetteSep) and then, PBMC profile was studied by flow cytometer plotting the forward-scatter (FSC) and side-scatter (SSC) parameters based on previous reported studies.

As expected, the red blood cell lysis buffer did not deplete any PBMC population (Figure S5A). After the white blood cell processing using Lymphoprep, the granulocyte population is removed, but the lymphocytes and monocytes remain (Figure S5B). In the RosetteSep condition it was only observed the lymphocyte fraction (Figure S5C).

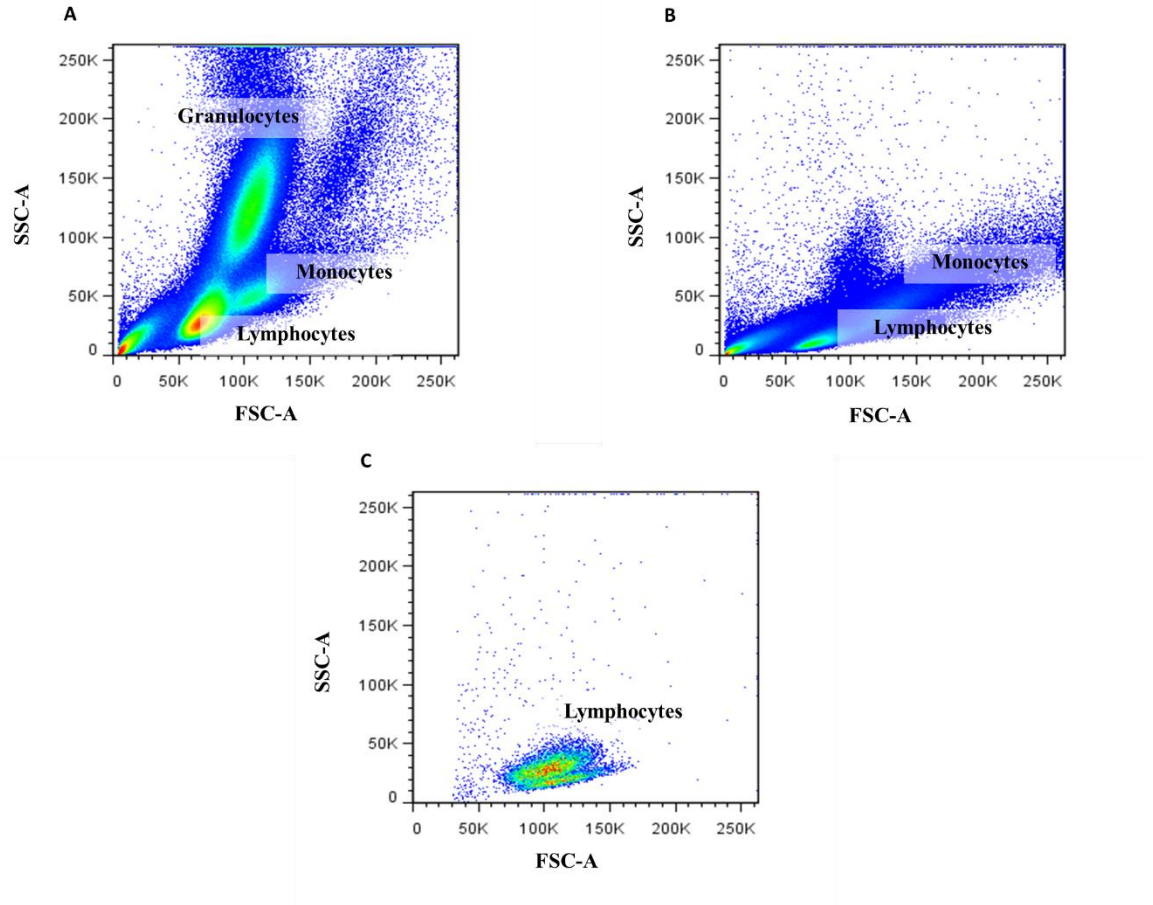


Figure S5. Isolation methods used to isolate PBMCs from HR+/HER2- stage IV BC patients. A) Red blood cells lysis buffer, B) Lymphoprep and C) RosetteSep (n= 1 replicate). PBMC profile was studied by flow cytometer plotting the forward-scatter (FSC) and side-scatter (SSC) parameters.

Considering the aforementioned results, the next step was confirmed that lymphocytes were the immune cell type enriched in the CTC fraction obtained after the isolation with RosetteSep. For that, blood samples from 10 randomly HR+/HER2- stage IV BC patients were processed with

RosetteSep (4 patients without previous therapy, 2 patients after one-cycle of CDK4/6i plus ET and 4 patients who progressed to 5th therapy line). Then, the CTC-enriched fraction was analysed in the flow cytometer as previously described. It was found that the main PBMC population that remained after the RosetteSep was the lymphocyte fraction, although a small percentage of monocytes was detected (FigureS6 A). The presence of lymphocytes and monocytes did not correlate with therapy line or the visit (FigureS6 B-E).

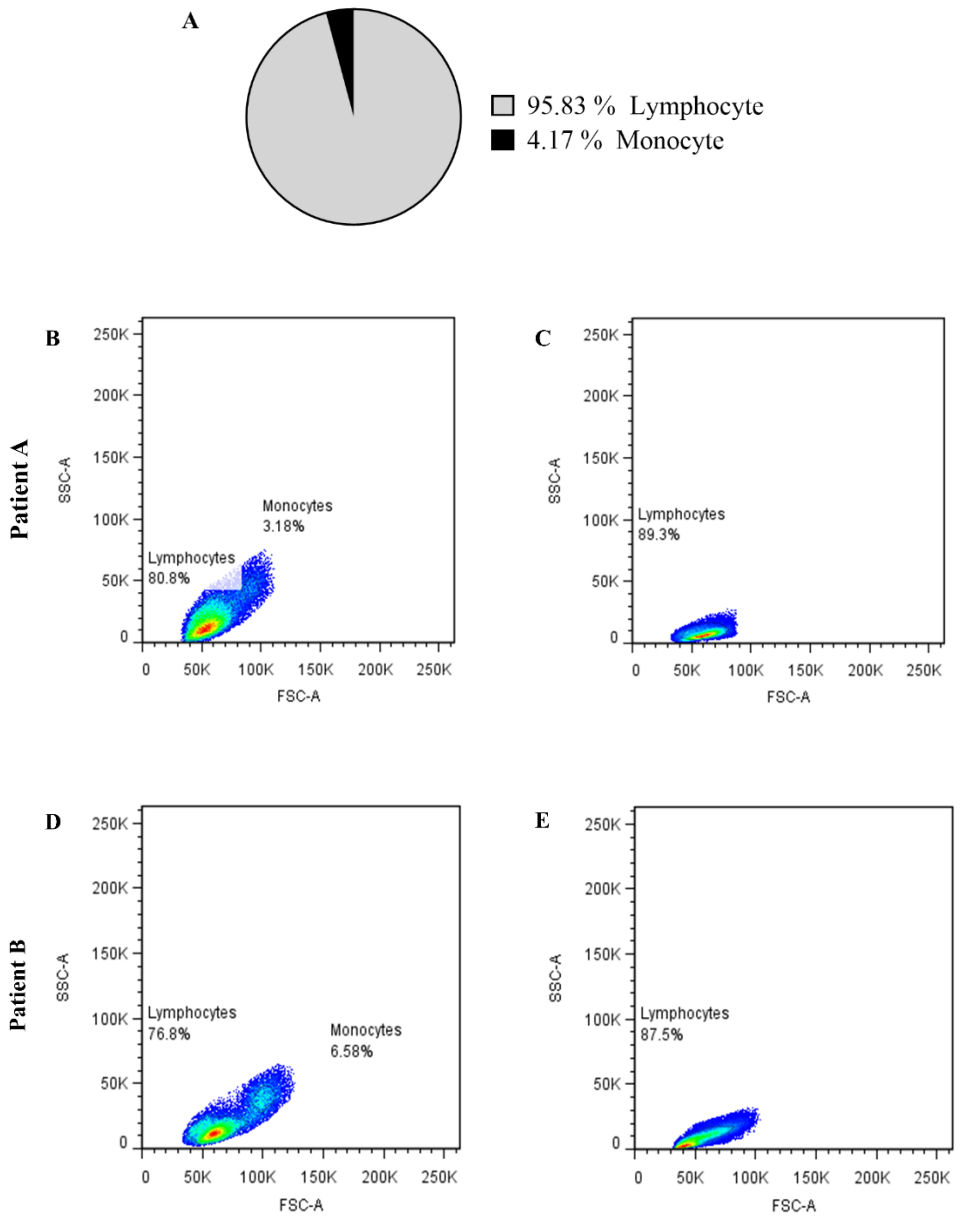


Figure S6. Analysis of PBMCs. A) Percentage of lymphocytes and monocytes in 10 HR+/HER2- stage IV BC patients analyzed by flow cytometer after RosetteSep. B-C) Percentage of lymphocytes and monocytes in patient A at visit 1 and 2 and D-E) Percentage of lymphocytes and monocytes in patient B at visit 1 and 2. PBMC profile was studied by flow cytometer plotting the forward-scatter (FSC) and side-scatter (SSC) parameters.

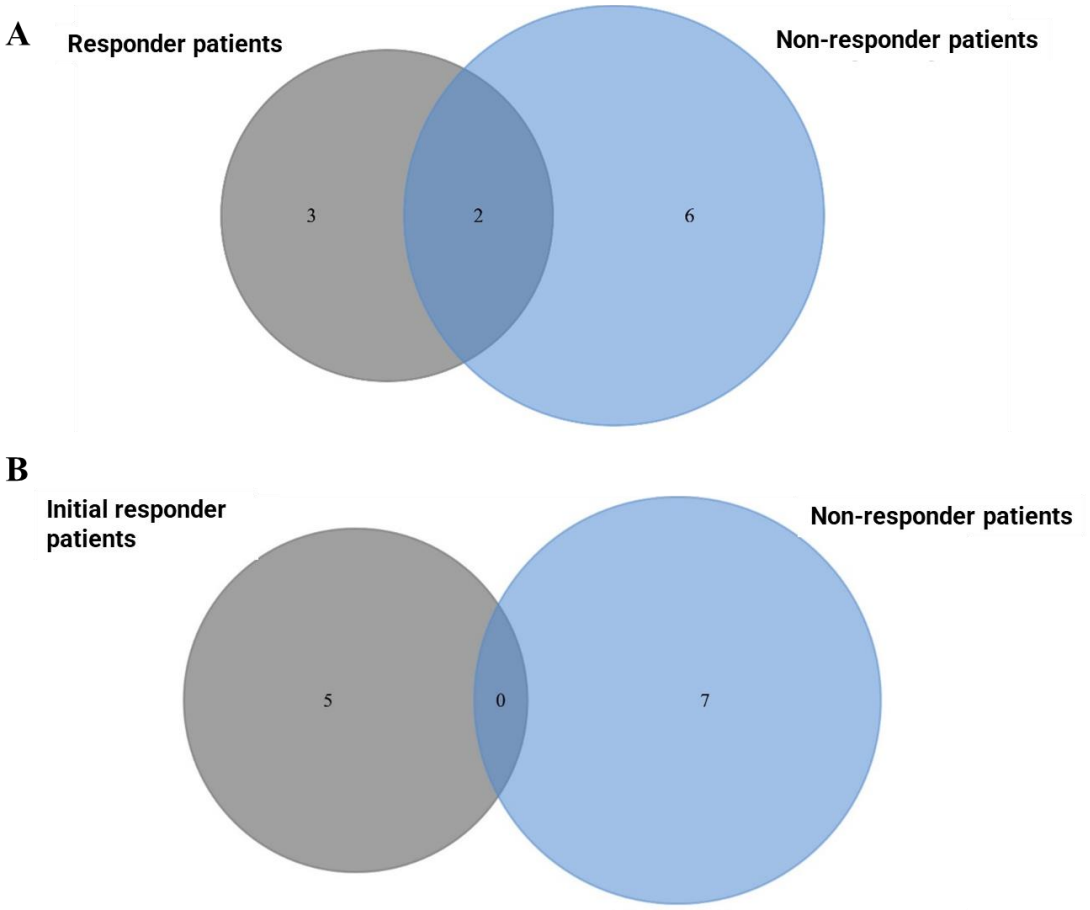
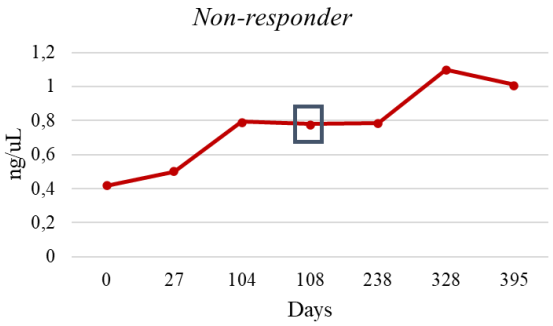
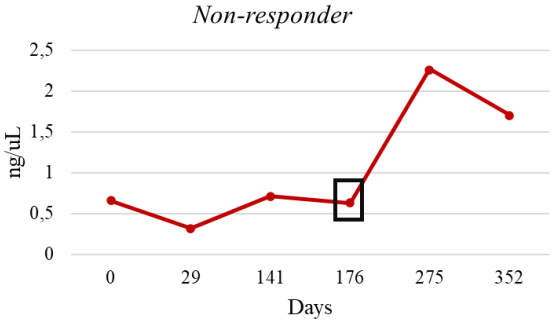
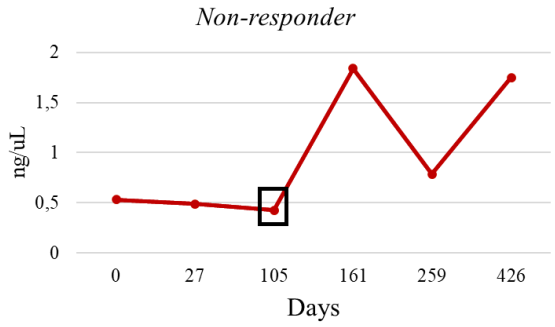
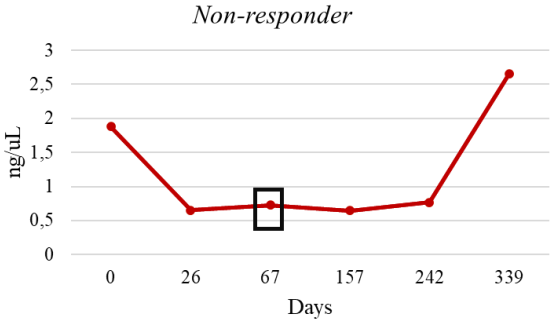


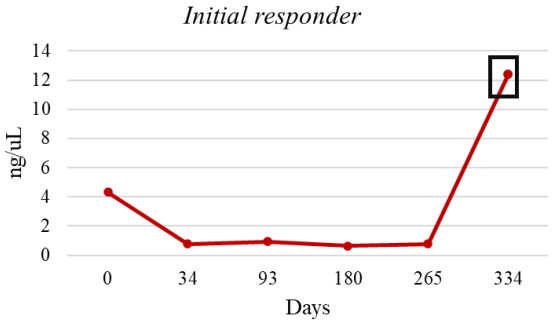
Figure S7. Venn's diagram depicting the mutations identified in the CTCs' DNA from HR+/HER2- stage IV BC patients after a DNA-seq analysis. A) Mutation identified at visit 1 in responder (initial and long responder) and non-responder patients and B) Mutations identified at visit 3 in initial and non-responder patients.

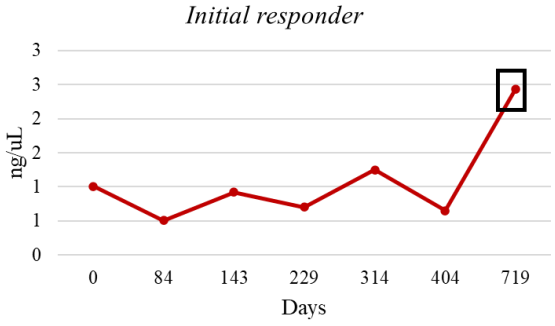


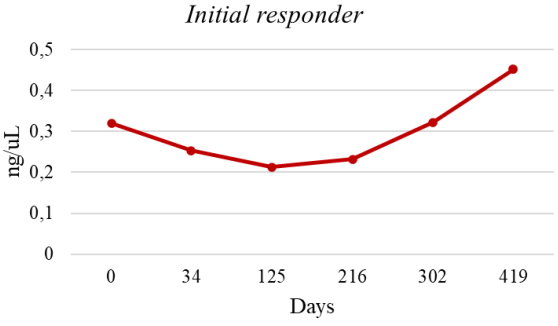


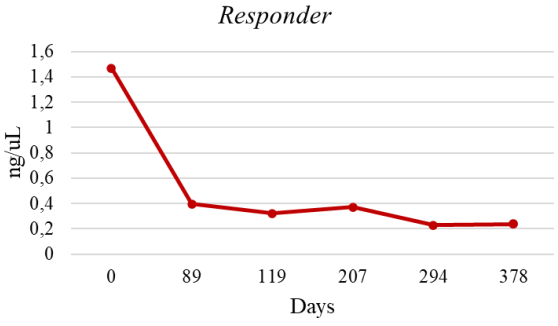


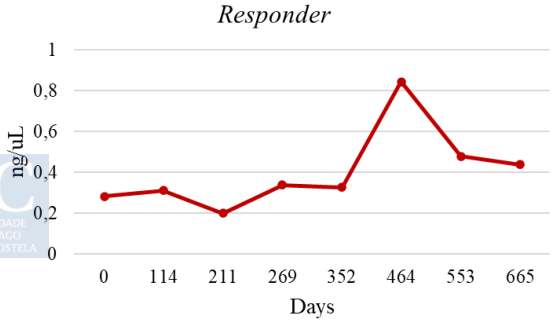












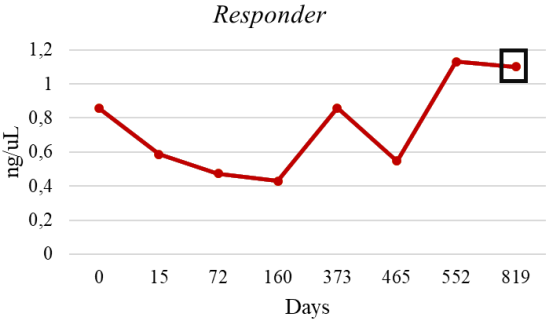


Figure S8. Longitudinal study of cfDNA concentration from 10 HR+/HER2- stage IV BC patients. cfDNA was isolated at visit 1, visit2, visit 3 and every 3 months up to 12 or 24 months. The square mark points the day when the disease progression was diagnosed. The disease progression sample from one initial patient was not collected.

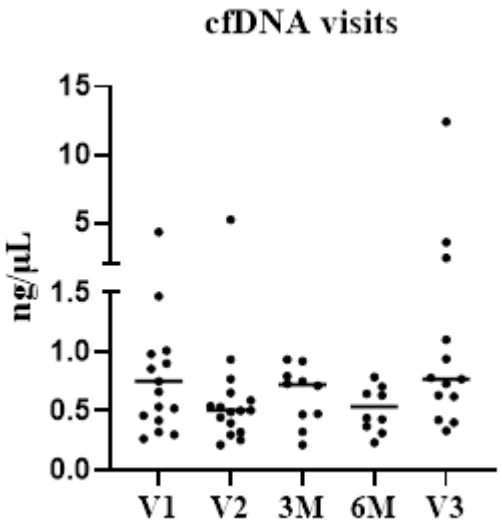


Figure S9. No statistical differences were found in the cfDNA concentration at different time points (n=21, visit 1), after one-cycle therapy (n=20, visit 2), at 3 and 6 months (n=10) and at progression (n=16, visit 3). (Mean 1.03 and SD 0.98 at visit 1; mean 0.85 and SD 1.18 at visit 2; mean 0.63 and SD 0.24 at 3M, mean 0.63 and SD 0.45 at 6M and Mean 1.9 and SD 3.2 at visit 3).

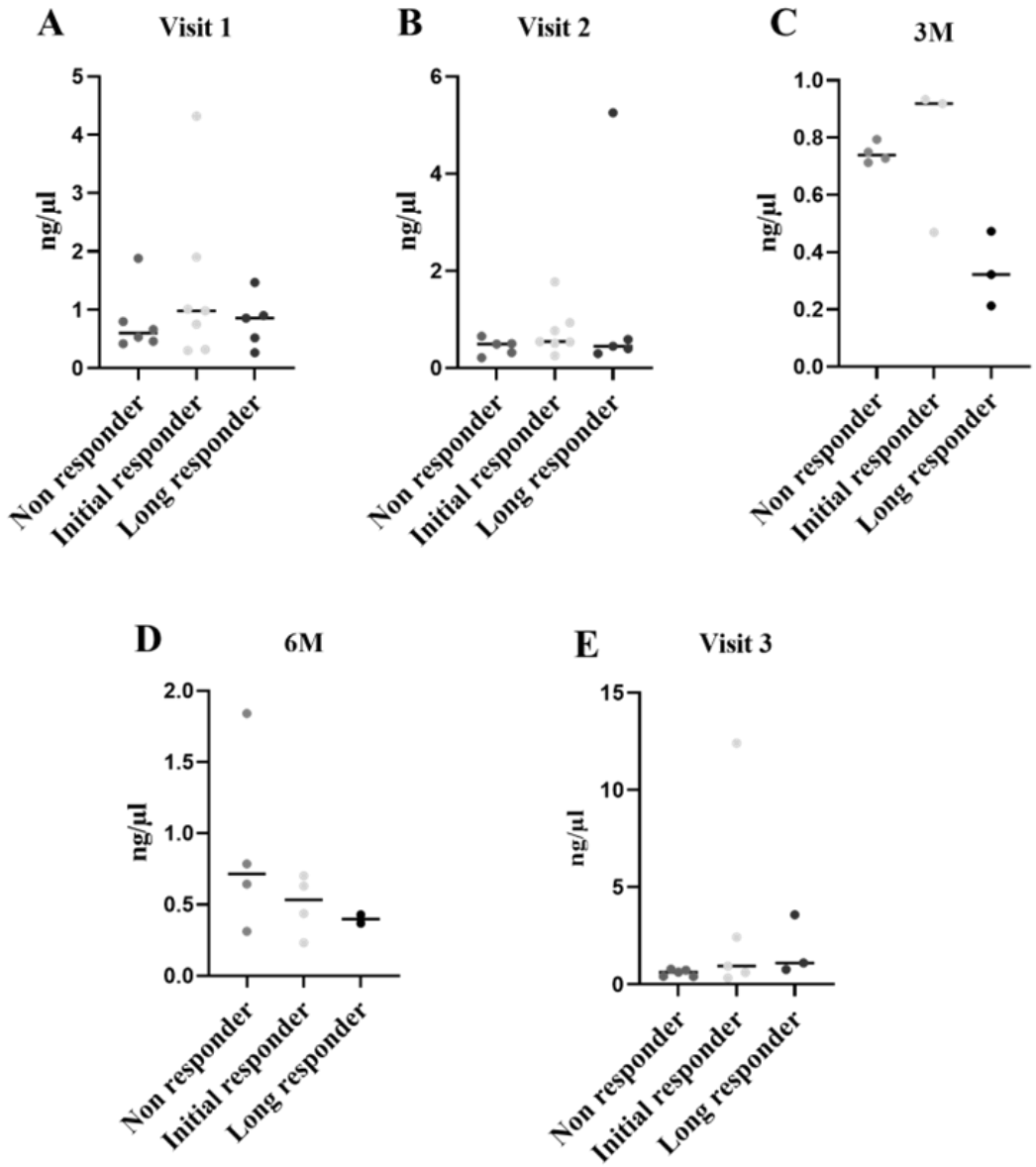


Figure S1022. No statistical differences were found in cfDNA concentration among patient groups at visit 1(n=6 NR, 7 IR, 5 LR), visit 2 (n=5 NR, 7 IR, 5 LR), 3M: 3 months (n=4 NR, 3 IR, 3 LR), 6M: 6 months (n=4 NR,4 IR, 3 LR) and visit 3 (n=5 NR, 5 IR, 3 LR). (NR: non-responder, IR: initial responder and LR: long responder).

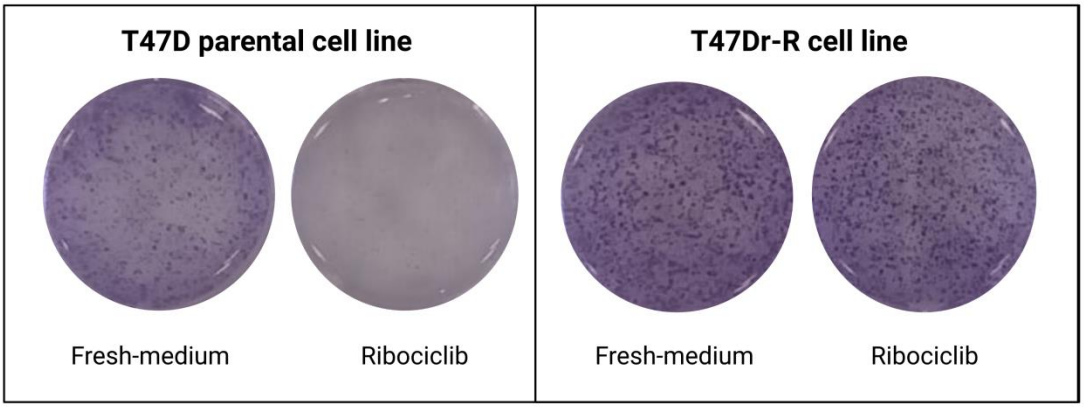


Figure S11. T47D colony formation assay. T47Dr-R and its counterpart were cultured with fresh-DMEM or Ribociclib for 1 week. Then, cells were cultured another week and colonies were counted by violet crystal staining.

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AGRADECIMIENTOS

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ANNEXES

XUNTA DE GALICIA
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DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 22/12/15 :

Título: Biopsia líquida para oncología de precisión
Promotor*:* Rafael López López
Tipo de estudio:EPA-SP
Version: versión 30 de noviembre de 2015 y HIP/CI (cultivo, control biopsia, y biopsia líquida) de la misma fecha
Código del Promotor:RLL-BL-2015_01
Código de Registro: 2015/772

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
C.H. Universitario de Santiago	Clotilde Costa Nogueira

XUNTA DE GALICIA
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Secretaría Xeral Técnica

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Teléfono: 881546425
ceic@sergas.es

galicia

Y HACE CONSTAR QUE:

1 El CAEIG cumple los requisitos legales vigentes (R.D 223/2004 por el que se regulan los ensayos clínicos con medicamentos, y la Ley 14/2007 de Investigación Biomédica).
2 El CAEIG tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del CAEIG es:

Manuel Portela Romero. (Presidente). Médico Especialista en Medicina Familiar y Comunitaria.
Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Juan Vázquez Lago (Secretario Suplente). Médico Especialista en Medicina Preventiva y Salud Pública.
Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Juan Casariego Rosón. Médico Especialista en Cardiología.
Xoán X. Casas Rodríguez. Médico Especialista en Medicina Familiar y Comunitaria.
Juana Mª Cruz del Río. Trabajadora Social.
Juan Fernando Cueva Bañuelos. Médico Especialista en Oncología Médica.
José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería
Pablo Nimo Ríos. Licenciado en Derecho. Miembro externo
Pilar Gayoso Diz. Médico Especialista en Medicina Familiar y Comunitaria.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Salvador Pita Fernández. Médico Especialista en Medicina Familiar y Comunitaria.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Susana María Romero Yuste. Médico Especialista en Reumatología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 05 de enero de 2015

SERVIZO GALEGO DE SAÚDE

DICTAMEN DEL Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

2015/772

Paula M. López Vázquez, Secretaria del Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

CERTIFICA:

Que este Comité evaluó en su reunión del día 27/06/23 la modificación del estudio**:**

Título: Biopsia líquida para oncología de precisión
Versión modificación*:* protocolo Versión 5: 15 de Junio de 2023
Tipo de estudio: EPA-SP
Promotor*:* Rafael López López
Código del Promotor: RLL-BL-2015_04
Código de Registro: 2015/772

Y que este Comité acepta de conformidad con sus procedimientos normalizados de trabajo y tomando en cuenta los requisitos éticos, metodológicos y legales exigibles, que dicha enmienda sea incorporada al estudio de investigación.

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SERVIZO GALEGO DE SAÚDE

Y HACE CONSTAR QUE:

1 El comité cumple los requisitos legales vigentes aplicables a los Comités de ética de investigación.
2 El comité tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del comité es:

Susana María Romero Yuste (Presidenta). Médico Especialista en Reumatología.
Diego Santos García (Vicepresidente). Médico Especialista en Neurología.
Paula Mª López Vázquez (Secretaria). Médico Especialista en Farmacología Clínica.
Martina Lema Oreiro (Vicesecretaria). Farmacéutica de Hospital.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Nuria Carballeda Feijóo. Miembro lego. Representante de los intereses de los pacientes.
Juana Mª Cruz del Río. Trabajadora Social.
Rafael Álvaro Millán Calenti. Asesor jurídico
José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Jorge Prado Casal. Licenciado en CC. Físicas. Experto en Protección de Datos.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Rafael Carlos Vidal Pérez. Médico Especialista en Cardiología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.
Irene Zarra Ferro. Farmacéutica de Hospital

Firmado digitalmente en Santiago de Compostela

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Frau
Prof. Dr. Sabine Kasimir-Bauer
Klinik für Frauenheilkunde und Geburtshilfe
- im Hause -

TRANSLATION OF THE EC APPROVAL
DATED 18.02.2013

Essen, den 24. Juni 20 / SB

Studientitel: **Molekulare Charakterisierung von zirkulierenden Tumorzellen (CTCs) als Ziele für die personalisierte Behandlung von metastasiertem Brustkrebs**
Antragsteller: **Prof. Dr. Kasimir-Bauer, Prof. Dr. Kimmig, Klinik für Frauenheilkunde und Geburtshilfe; Prof. Dr. Schuler, Innere Klinik (Tumorforschung)**
Unser Zeichen: **12-5265-BO**

Sehr geehrte Frau Prof. Kasimir-Bauer,
dear Prof. Kasimir-Bauer,

die Ethik-Kommission der Medizinischen Fakultät der Universität Duisburg-Essen hat Ihren o. g. Antrag geprüft.
the Ethical Review Board at the Medical Faculty of the University Duisburg-Essen has proved your application with the above study title.

Auf der Grundlage der übersandten Unterlagen vom 20.09.2012 und 13.02.2013 und dem derzeitigen Informationsstand sieht die Ethik-Kommission keinen Anlass, ethische oder rechtliche Einwände gegen diese Studie zu erheben.
Based on the provided documents dated 20.09.2012 and 13.02.2013 and the current state of information there are no ethical or legal objections regarding your study.

Die Verantwortung für die Studie und ihre Durchführung verbleibt uneingeschränkt bei den Prüfärzten und wird nicht von der Ethik-Kommission übernommen.
The Ethical Review Board is not responsible for the study and its execution.

Mit freundlichen Grüßen
Yours sincerely,

Prof. Dr. U. Schara-Schmidt

All figures have been created by the author using Biorender.com, except for Figure 1 and 6.

Figure 1 was adapted from one published article “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries” by Hyuna Sung, Jacques Ferlay, Rebecca L. Siegel, Mathieu Laversanne, Isabelle Soerjomataram, Ahmedin Jemal and Freddie Bray, with permission of the publishers.


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Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries

Hyuna Sung PhD, Jacques Ferlay MSc, ME, Rebecca L. Siegel MPH, Mathieu Laversanne MSc, Isabelle Soerjomataram MD, MSc, PhD, Ahmedin Jemal DMV, PhD, Freddie Bray BSc, MSc, PhD

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Figure 6 was adapted from one published article “ESMO Clinical Practice Guideline for the diagnosis, staging and treatment of patients with metastatic breast cancer” by A Gennari, F André, C.H. Barrios, J. Cortés, E. de Azambuja, A. DeMichele, R. Dent, D. Fenlon, J. Gligorov, S. A. Hurvitz , S-A Im , D. Krug , W.G. Kunz , S. Loi , F. Penault-Llorca, J. Ricke, M Robson, H.S. Rugo, C. Saura, P. Schmid, C.F. Singer, T. Spanic, S. .M Tolaney, N.C. Turner , G. Curigliano, S. Loibl, S. Paluch-Shimon, N. Harbeck, with permission of the publishers.


ESMO Clinical Practice Guideline for the diagnosis, staging and treatment of patients with metastatic breast cancer

A Gennari [1], F André [2], C H Barrios [3], J Cortés [4], E de Azambuja [5], A DeMichele [6], R Dent [7], D Fenlon [8], J Gligorov [9], S A Hurvitz [10], S-A Im [11], D Krug [12], W G Kunz [13], S Loi [14], F Penault-Llorca [15], J Ricke [16], M Robson [17], H S Rugo [18], C Saura [19], P Schmid [20], C F Singer [21], T Spanic [22], S M Tolaney [23], N C Turner [24], G Curigliano [25], S Loibl [26], S Paluch-Shimon [27], N Harbeck [28]; ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org

Affiliations + expand

PMID: 34678411 DOI: 10.1016/j.annonc.2021.09.019

Free article

HR+/HER2- stage IV breast cancer patients are treated with a combination of CDK4/6 inhibitors plus endocrine therapy (ET). Nevertheless, all patients will develop intrinsic or acquired resistance, but there are not predictive biomarkers identified so far. Hence, in this thesis, it was proved that the study of circulating material lets monitor disease evolution and tailor-patient therapy. The transcriptomic characterization of CTCs classified patients according to therapy response and determine resistance mechanisms to CDK4/6i plus ET. The cfDNA levels can be used to longitudinally study therapy response. In addition, it was proposed the characterization of circulating immune cells to identify predictive biomarkers to CDK4/6i plus ET in HR+/HER2- stage IV BC patients.